Expanding the toolkit of the *Chlamydomonas reinhardtii* chloroplast for biotechnological applications

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A thesis submitted for the degree of *Doctor of Philosophy*

April 2016

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I. Declaration

I hereby declare that the work presented in this thesis with the title "Expanding the toolkit of the *Chlamydomonas reinhardtii* chloroplast for biotechnological applications" was carried out by me and is original unless stated otherwise where specific reference is made. If work was carried out by collaborators, it is specified in the author contribution section of the respective chapter. None of this work has been submitted previously for another degree or qualification at this or any other university.

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement no. 317184. This material reflects only the author's view and the Union is not liable for any use that may be made of the information contained therein.

Julie A. Z. Zedler

Canterbury, 5th April 2016

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IV. List of abbreviations

AA – amino acid

ATP – adenosine triphosphate

CHO – Chinese hamster ovary

CoA – coenzyme A

DHA – docosahexaenoic acid

DMAPP – dimethylallyl pyrophosphate (dimethylallyl diphosphate)

DNA – deoxyribonucleic acid

DTT – dithiothreitol

DXS – deoxy-xylulose-5-phosphate synthase

EDAB – ecosystem disruptive algal bloom

EDTA – ethylenediaminetetraacetic acid

EPA – eicosapentaenoic acid

ER – endoplasmic reticulum

EUR – euros

Fd – ferredoxin

FISH – fluorescence in situ hybridisation

FMDV – foot-and-mouth-disease-virus

FNR – ferredoxin $NADP^+$ reductase

GA3P – glyceraldehyde-3-phosphate

G+C – guanine and cytosine

GC-MS – gas chromatography-mass spectrometry

GGPP – geranylgeranyl pyrophosphate (geranylgeranyl diphosphate)

GGPPS – geranylgeranyl pyrophosphate synthase

GGW – glass green wall

GOI – gene of interest

GM – genetically modified

GMO – genetically modified organism

GRAS – generally regarded as safe

HAB – harmful algal bloom

HBsAg – hepatitis B surface antigen

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGT – horizontal gene transfer

HMG – 3-hydroxy-3-methyl-glutaryl

HPLC – high performance liquid chromatography

HSM – high salt medium

IM – inner envelope membrane

IPP – isopentenyl pyrophosphate (isopentenyl diphosphate)

Kb – kilo base

kDa – kilo Dalton

LSU – RuBisCO large subunit

MALDI-TOF – matrix assisted laser desorption ionisation time-of-flight

Mb – mega base

MeOH – methanol

MEP– methyl-D-erythritol 4-phosphate

MS – mass spectrometry

M-SAA – bovine mammary-associated serum amyloid

MVA – mevalonate

NADH – nicotinamide adenine dinucleotide (reduced)

NADPH – nicotinamide adenine dinucleotide phosphate (reduced)

OM – outer envelope membrane

P450 – cytochrome P450 (monooxygenase)

PBR – photobioreactor

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PET – photosynthetic electron transport

PHB – poly-3-hydroxybutyrate

PSI – photosystem I

PTM – post-translational modification

PUFAs – polyunsaturated fatty acids

rpm – revolutions per minute

rRNA – ribosomal ribonucleic acid

RuBisCO – ribulose-1,5-bisphosphate carboxylase/oxygenase

S – stroma

scFv – single chain Fragment variable

SDS – sodium dodecyl sulphate

Sec – secretory

SPME – solid-phase microextraction

SRP – signal recognition particle

TAP – tris-acetate-phosphate

Tat – twin-arginine translocation

TCP – total cellular protein

Temp. – temperature

TIC – translocon at the inner chloroplast envelope

TL – thylakoid lumen

TM – thylakoid membrane

TOC – translocon at the outer chloroplast envelope

TPS4 – *cis*-abienol diterpene synthase (AbCAS)

TSP – total soluble protein

USD – US dollar

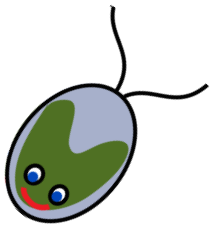
UTR – untranslated region

WT – wild type

µE – µmol photons m^{-2} s^{-1}

Ψ – electrical potential

V. Acknowledgments



I want to thank my PhD supervisor Colin Robinson for giving me the opportunity to take up this PhD project, his support, advice and also for letting me follow my own ideas during this process even if they didn't always turn out to be a sensible option.

Thank you to all ex- and current lab members of the Colin Robinson group at the University of Kent who made sure there was coffee and biscuits to keep me going through all ups and downs in the last three years and created a very friendly and fun working environment.

Thank you to all members of the PHOTO.COMM consortium, especially my second supervisor, Patrik Jones for useful discussions and critical thoughts. I also want to thank Tiago Guerra and the whole team at A4F for a very productive time during my secondment at the company in Lisbon; Marc Scaife and Alison Smith for hosting me during a secondment at the University of Cambridge and Poul Erik Jensen, Artur Włodarczyk, Erick Ramos Martinez, Kamil Bakowski, Trine Bundgaard Andersen, Irini Pateraki, Björn and Britta Hamberger for productive collaborations between Kent and Copenhagen and for hosting me several times.

I also want to thank Saul Purton for setting me up with the basics of *Chlamy* transformations that were crucial for a successful start into my PhD, Conrad Mullineaux for teaching me the basics of confocal imaging, Kevin Howland for helping me out with several experiments involving HPLC, GC and MS at Kent and all my co-authors on the publications that resulted from the last three years of work.

Many thanks also go to my family, especially my mum, for their support and for never doubting that I would manage what I was planning, even if it involved moving to a different country and quitting a degree to start my PhD and pursue what I decided was right. I also want to thank David Russo, for countless discussions involving far too many algae at any time of the day, a lot of patience, unconditional support and also for proofreading my thesis.

Thank you to the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ for funding this research under REA grant agreement no. 317184.

VI. Summary

Microalgae have gained increasing interest over the last ten years for their exploitation for biotechnological applications. A commonly used model organism is *Chlamydomonas reinhardtii* and many recombinant proteins have been successfully expressed in this green microalga. The chloroplast is of special interest as it allows rather high expression levels and targeted gene integration. Tools for chloroplast transformation and expression systems have advanced remarkably over the last years and the field is developing increasingly faster.

In this thesis, existing tools for genetic modification of *C. reinhardtii* were used to expand further the toolkit, mainly for the synthesis of high-value diterpenes of plant origin, and to examine the potential of using these microalgae in an industrial setting. A strain expressing a large recombinant enzyme, a diterpene synthase, resulting in *in vivo* synthesis of the diterpene *cis*-abienol, was generated. The potential for light-driven product synthesis via a chloroplast re-located cytochrome P450 was successfully shown. It was also demonstrated that typically fragile, wall-deficient transgenic strains can be grown on a 100 L pilot scale. This is an important finding as the gap between lab- and large-scale studies needs to be closed to allow a transition to an industrial setting. It was also shown that there is still untapped potential in the algal chloroplast – by utilising a bacterial export signal peptide the chloroplast toolkit was expanded to the thylakoid lumen for recombinant protein production.

Three proof of concept studies presented herein and one study looking at pilot scale cultivation of previously generated transgenic strains show that the potential seen in microalgae for biotechnological applications is justified and further developments and improvement of the existing strains could make this a viable competitive platform for diverse applications.

Chapter 1: Introduction – genetic engineering and biotechnological applications of microalgae

1.1. The model organism *Chlamydomonas*

Algae are a polyphyletic class of significant diversity. Currently organisms considered to be algae belong to four different kingdoms. The definition of "an alga" is therefore a rather broad term. Michael D. Guiry gives a definition that is widely accepted by phycologists:
"…generally algae are considered to be aquatic, oxygen-evolving photosynthetic autotrophs that are unicellular, colonial or are constructed of filaments or composed of simple tissues." (Guiry, 2012).

All experiments of this PhD thesis were performed using *Chlamydomonas reinhardtii*. Macroalgae will be excluded as it is far beyond the scope of this thesis. If appropriate, references to observations and studies in cyanobacteria will be given. In this thesis the focus will be on eukaryotic microalgae, in particular green algae – the direct relatives of the organism used in this thesis. *C. reinhardtii* has a history as a model organism for many decades. Most of the commonly used lab strains are thought to originate from isolates back in 1945 (Harris, 2001). *C. reinhardtii* is a biflagellate, unicellular chlorophyte with a large single chloroplast. The alga has a fast doubling time, is easy to grow and it can reproduce sexually and asexually. Cultivation is possible under various regimes from photoautotrophic over mixotrophic to heterotrophic. All these features make it a popular model organism of choice in various fields of research. In more recent years it was increasingly subjected to genetic modification studies. Compared to other microalgae, we know a lot about its physiology and metabolism (due to its history as a model organism) enabling us to introduce foreign metabolic pathways and investigate its use for novel applications. This is also the reason why *C. reinhardtii* was the organism of choice for the studies described in this PhD thesis.

This introduction will give a broad overview of the current and potential future exploitation of microalgae. A focus is set on high-value compound synthesis by engineering recombinant proteins (pathways) into the alga. The state of the art of genetic modification tools for microalgae (with a focus on *Chlamydomonas* sp.) will be summarised. Potential applications and the current situation of large-scale cultivation systems and markets for algal products will be discussed.

1.2. Exploitation of microalgae

1.2.1. Historical use of microalgae

The first known use of microalgae in human culture was approximately 2000 years ago, when the cyanobacterium *Nostoc* sp. was consumed, in China, for food due to famine (Spolaore *et al.*, 2006). Also, other cyanobacteria such as *Spirulina* sp. and *Aphanizomenon* sp. have been used for food purposes over thousands of years (Jensen *et al.*, 2001). The consumption of algae as food or nutritional supplement is fairly common in Asia (Pryadarashni and Rath, 2012), but a more recent occurrence in the rest of the world. Another microalgae traditionally used for food purposes is the green alga *Chlorella*. Around the time of the first and second world war it became popular due to its putative power to solve problems of hunger and an increasing world population (given its nutritional value and high protein content) (Belasco, 1997) and algal mass cultivation systems started to develop (Walker *et al.*, 2005). In the middle of the last century the development of microalgae for biotechnological applications started (Spolaore *et al.*, 2006).

1.2.2. Modern applications of microalgae

This section is divided into three major fields of applications for microalgae. First, the potential use of algae for commodity – more specifically biofuels – production is discussed, then the production of in algae naturally abundant high-value compounds will be introduced. Finally, an overview of why microalgae are considered a promising host for industrial biotechnology is given.

1.2.2.1. Microalgae for biofuel production

Research on microalgae for oil production has started several decades ago (Oswald and Golueke, 1960). In 1978, the U.S. Department of Energy started the "Aquatic Species Program" to investigate the potential of over 150 microalgal species as a renewable energy source. This project was, however, terminated in 1996 (Sheehan

et al., 1998). In the year 2007 these plans of biofuel production from microalgae were revived, most likely due to an increase in oil price. A review paper that claimed microalgae could have up to 70 % of oil content (by weight) and would be the only crop for the future capable of replacing fossil fuels for biodiesel production probably played a major role in this revival (Chisti, 2007). Up to October 2015 this paper has been cited nearly 3000 times according to Web of Science™. However, the numbers mentioned in the paper have been doubted many times and biodiesel production from microalgae is still not feasible. A lot more research followed up on this claim and the field has since progressed substantially. Generally, microalgae are still regarded as one of the most promising crops for biofuel production (Wijffels and Barbosa, 2010).

The idea of using microalgae for biofuels is plausible – fossil fuels are finite and alternative, sustainable energy sources have to be found to satisfy increasing demand and tackle climate change. Currently, biofuels are mainly made from plants, but there is increasing concern with the use of edible crops for fuel production (Georgianna and Mayfield, 2012; Adenle *et al.*, 2013). These "first generation biofuels" have several more problems such as low-land use efficiency, poor carbon balance and competition for arable land (Schenk *et al.*, 2008; Andersson *et al.*, 2014). Microalgae have several advantages over terrestrial crops such as cultivation on non-arable land, year round cultivation and higher productivities (Moody *et al.*, 2014). There is even potential to grow algae for biofuel production on wastewater (reviewed for Chlorophyta in (Abinandan and Shanthakumar, 2015)).

In general, three different fuels can be made from microalgae: biodiesel (via transesterification), petrol or jet fuel (Georgianna and Mayfield, 2012). This type of renewable jet fuel is made via transesterification of triacylglycerols and fatty acids with subsequent hydrocracking and hydroprocessing (Kallio *et al.*, 2014). Diatoms are considered to be one of the most promising candidates for biofuel production (Ramachandra *et al.*, 2009), but also many other microalgae have been considered.

A general consent between scientists is that biofuel production from microalgae needs considerable improvements such as an increase in productivity, reduction of production costs (including downstream processing and algal cultivation) and increase of lipid content. The potential to achieve this is given through several routes, including metabolic and genetic engineering of microalgal strains of interest, screening of natural isolates and selection of strains by adaptation (Schenk *et al.*, 2008). Another idea to increase the feasibility is a combined biorefinery approach where several different

products from the algal biomass can be extracted (Wijffels *et al.*, 2010; Markou and Nerantzis, 2013; Lee Chang *et al.*, 2014).

Estimations of how long it will take to develop algal biofuel production into an economically viable process vary substantially. The next five to ten years will show if a common effort could significantly improve the process and bring algae production of biofuels to a commercial scale. Not only further scientific and technical development will be needed, but, specifically for genetically modified strains, also policy and legislation hurdles will have to be overcome.

1.2.2.2. Microalgae for natural high-value compound production

In contrast to biofuels, natural high-value compounds are already produced in algae on an industrial scale. The molecules produced include carotenoids such as β-carotene (mainly produced in *Dunaliella salina*) (Borowitzka, 2013) and astaxanthin (mainly from *Haematococcus pluvialis* (Lorenz and Cysewski, 2000)), phycocyanin from cyanobacteria (produced in *Arthrospira platensis*, previously described as *Spirulina platensis*) (Borowitzka, 2013) or ω-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 fatty acids are produced from various hosts such as *Nannochloropsis* (an eustigmatophyte), *Crypthecodinium* (a dinoflagellate) (Mendes *et al.*, 2009), *Schizochytrium* and *Ulkenia*. Further interesting products are proteins, antioxidants and other pigments. Several of these high-value compounds are ingredients to a variety of pharmaceutical and cosmetic products (Gangl *et al.*, 2015a). *Spirulina* and *Chlorella* are also produced as feed, food additive and diet supplements directly sold in a dried form (Enzing *et al.*, 2014). The microalgal product global market value is estimated at 1.5 million US dollars per year worldwide (Verdelho Vieira, 2014).

In addition to high-value compounds that are already produced in microalgae, many more have been investigated at lab scale. Microalgae are considered to have considerable antioxidant properties and contain phenolic compounds of interest for pharmaceutical and food applications. The cyanobacterium *Fisherella ambigua* and the microalga *Chlorella vulgaris* have been highlighted for these properties (Hajimahmoodi *et al.*, 2010). Microalgae produce a variety of other compounds such as sterols,

polyhydroxyalkanoates, polysaccharides and further bioactive molecules with antibiotic, antiviral, anticancer, anti-inflammatory or antihypotensive properties (Borowitzka, 2013). For many of these products lab scale studies are available, but the feasibility of a transition to industrial production remains to be proven. This will also largely depend on market demands and improvements of biomass production costs for microalgae.

1.2.2.3. Potential of microalgae as a biotechnological production platform

Microalgae have great potential as a biotechnological host because of several reasons that will be discussed in the following section. Their diversity in cellular architecture and biosynthetic capacity (Scaife *et al.*, 2015) provides a versatile background for various applications in biotechnology. So called 'molecular farming' in algae has been discussed for many years as an attractive, "green" and sustainable alternative with future.

Most green algae (e.g. *Chlorella vulgaris* and *Chlamydomonas reinhardtii*) are classified under the GRAS (generally regarded as safe) status. This is a clear advantage for cultivation. Complying with environmental, as well as health and safety regulations, aids the development of edible products for food and feed from microalgae (Johanningmeier and Fischer, 2010). *Chlamydomonas* has previously been described as a 'planimal' referring to the mixture of traits from both, the plant and animal kingdoms (Redding and Cole, 2008) which can provide a valuable addition to industrial biotechnology. Another important feature is the compartmentalisation present in eukaryotic microalgae. The chloroplast, specifically, is of interest for protein and high-value compound production (Potvin and Zhang, 2010; Purton *et al.*, 2013). The chloroplast facilitates folding of recombinant proteins (Chebolu and Daniell, 2009) and relatively high gene expression levels can be achieved. Studies have reported levels of up to 10 % of total soluble protein (TSP) (Manuell *et al.*, 2007) or 20.9 ± 1.2 % total cellular protein (TCP) (Surzycki *et al.*, 2009). Regarding recombinant protein production, the ability of post-translational modification (PTM) is a further desirable trait for more complex protein production. The algal chloroplast is capable of disulphide bond formation (Kim and Mayfield, 1997; Mayfield *et al.*, 2003) and hosts many chaperones which assist protein folding (Schroda, 2004). Glycosylation does not occur

in the chloroplast (Mayfield and Franklin, 2005) but occurs in the cytosol even though our knowledge about the processes of glycosylation in microalgae is still very limited (Mathieu-Rivet *et al.*, 2014).

To date, microalgae are not properly established as a biotechnological host, although some researchers are convinced of the maturity of the platform (Specht *et al.*, 2010). Considerable progress has however been made over the last years and important milestones were reached. This is specifically the case for *C. reinhardtii*: all three genomes, the nuclear, mitochondrial and chloroplast genome, are sequenced (Vahrenholz *et al.*, 1993; Maul *et al.*, 2002; Merchant *et al.*, 2007), all genomes can be genetically manipulated and an impressive collection of mutants is available (Neupert *et al.*, 2009). Genetic modification techniques and achievements of high-value compounds produced in microalgae will be reviewed in more detail in section 1.3 and 1.4. with a focus on *C. reinhardtii*.

In summary, there is great potential of microalgae as cell factories in biotechnology, but the details of how to implement this are still not entirely clear. A widely spread opinion proposes microalgae as a good replacement for conventional and already established hosts due to their supposedly lower production costs (the idea is that algae can essentially be grown only with light and don't need many other resources). Other cell factories such as yeast or mammalian cell cultures have been advanced over many years according to the needs of biotechnology and are highly competitive. It seems more likely that a 'niche' for microalgae will have to be found. This niche would ideally complement other production platforms utilising unique microalgal traits and potentials instead of attempting to replace those.

1.3. Genetic modification techniques and tools in microalgae

The genetic modification of microalgae is an essential tool to develop microalgae for future exploitation, not only as cell factories in industrial biotechnology, but also for enhancing traits of algal metabolism (e.g. faster growth or higher lipid yields). To date, the transformation of over 40 different microalgal species has been reported (Gangl *et al.*, 2015a). Most reports of successful transformation are found for *C. reinhardtii*, although transformation systems are also advanced for the diatom

Phaeodactylum tricornutum, several *Chlorella* species and *Volvox carteri* (Walker *et al.*, 2005), and progress has been made in species such as *Haematococcus pluvialis* (Sharon-Gojman *et al.*, 2015), *Nannochloropsis* (Cha *et al.*, 2011; Kilian *et al.*, 2011) and *Schizochytrium* (Cheng *et al.*, 2012). *Chlamydomonas* is a widely preferred organism of choice for genetic modification studies in microalgae, not only because of the fairly established tools available, but also because stable transformation lines can be generated in a comparably short timeframe of a few weeks from gene design to scale up (Mayfield *et al.*, 2007).

In the following section the basics of genetic modification for eukaryotic microalgae will be reviewed with a focus on recombinant proteins, DNA delivery methods, integration sites for the transgene and their advantages and disadvantages and examples of promoters, reporter genes and selectable markers available. The focus will be on *C. reinhardtii* since this is most relevant for this thesis and the techniques and tools are the most advanced, but systems in other microalgae will also be introduced.

1.3.1. DNA delivery methods

The transformation of microalgae is not a novelty as such – the first successful transformation dates back more than 30 years when a yeast DNA fragment encoding *arg4* was expressed in *C. reinhardtii* to complement arginine auxotrophy (Rochaix and van Dillewijn, 1982). The first successful transformation of the *C. reinhardtii* chloroplast genome was reported in 1988 using the method of tungsten microparticle bombardment (biolistics) (Boynton *et al.*, 1988). Shortly afterwards it was shown that this method can also be used to transform the nuclear genome (Debuchy *et al.*, 1989). Biolistics have proven to be a very popular way of DNA delivery and have also been used to transform other microalgae such as *Phaeodactylum tricornutum* (Apt *et al.*, 1996; Falciatore *et al.*, 1999), *Haematococcus pluvialis* (Teng *et al.*, 2002; Sharon-Gojman *et al.*, 2015), *Nannochloropsis* sp. (Kilian *et al.*, 2011) and *Chlorella sorokiniana* (Dawson *et al.*, 1997).

Only two years after biolistics were introduced for *Chlamydomonas* transformation, an alternative method by agitation of a cell and DNA mixture with glass beads was developed. First, the nuclear genome was targeted (Kindle, 1990) and shortly

afterwards the method was also shown to work for modifying the chloroplast genome (Kindle *et al.*, 1991). The basic principle behind this transformation method is the generation of transient holes in the cell by the glass beads where the DNA can then be incorporated into the cell interior.

These two methods are still the most commonly used ones to deliver DNA for *Chlamydomonas* transformation and the techniques have been optimised and matured over the years. Whereas the glass bead method has a lower transformation efficiency, the particle bombardment method needs expensive equipment not available to every researcher. Cell wall deficient strains are routinely used for transformation (Potvin and Zhang, 2010), especially with the glass bead method. Alternatively, the *Chlamydomonas* cell wall can be removed with autolysin prior to the transformation procedure (Kindle, 1990).

A third common DNA delivery method is via electroporation (Brown *et al.*, 1991). Transformation efficiencies for this method were initially rather poor, but later improvements made this method a respectable alternative to particle bombardment and the glass bead method in *Chlamydomonas* (Tang *et al.*, 1995; Shimogawara *et al.*, 1998). Electroporation has also been used for nuclear (Miyahara *et al.*, 2013; Zhang and Hu, 2014) and chloroplast transformation (Xie *et al.*, 2014) of the diatom *P. tricornutum* and other microalgae such as *Chlorella vulgaris* (Chow and Tung, 1999; Niu *et al.*, 2011) and *Dunaliella salina* (Geng *et al.*, 2003).

In addition to the classical delivery procedures, other methods have been explored over the years. One transformation method, which is very well established in plants, is mediated by *Agrobacterium tumefaciens.* This system has been used to transform several algae including *C. reinhardtii* (Kumar *et al.*, 2004; Pratheesh *et al.*, 2014), *Haematococcus pluvialis* (Kathiresan *et al.*, 2009), *Chlorella vulgaris* (Cha et al., 2012), *Nannochloropsis* sp. (Cha *et al.*, 2011) and *Schizochytrium* sp. (Cheng *et al.*, 2012). Silicon carbide whiskers have also been tested to transform *Chlamydomonas* (Dunahay, 1993) and dinoflagellates (ten Lohuis and Miller, 1998), but this method, to date, has not been further established. This might also be due to inhalation hazards associated with the silicon carbide whiskers (Qin *et al.*, 2012). The most recently reported new method uses positively charged nanoparticles coated with the DNA for transformation (Kim *et al.*, 2014). Moreover, this method can be used to transform the nuclear genome of walled *C. reinhardtii* strains and is supposedly more efficient, cheaper and easier than particle bombardment.

Other DNA delivery methods that have been used, and are proposed to have potential for further application in microalgae, are microinjection and use of an artificial transposon (Qin *et al.*, 2012). To sum up, several methods have been developed for the transformation of microalgae over the years. Efficiencies of transformation have been improved, but DNA cannot always be integrated at a specific site meaning that it integrates randomly which is a major constraint. This will have to be addressed in future research; details of this will be discussed in the next section for *Chlamydomonas*.

1.3.2. Integration of transgenes: nuclear vs. chloroplast genome

The mechanisms of how a transgene can be integrated into the targeted genome can vary significantly between microalgal species, genomes targeted and transformation methods used. In theory, three different genomes can be targeted in eukaryotic microalgae: the nuclear genome, the chloroplast genome and the mitochondrial genome. In the case of *C. reinhardtii*, transformation of all three genomes has been reported (Boynton *et al.*, 1988; Debuchy *et al.*, 1989; Randolph-Anderson *et al.*, 1993). Fig. 1.1. gives an overview of the targeted integration sites and advantages and disadvantages each gene integration and expression site bears (especially for more complex recombinant protein expression). In most cases, either the nuclear or the chloroplast genomes have been targeted. In the following sections these will both be discussed in more detail and compared.

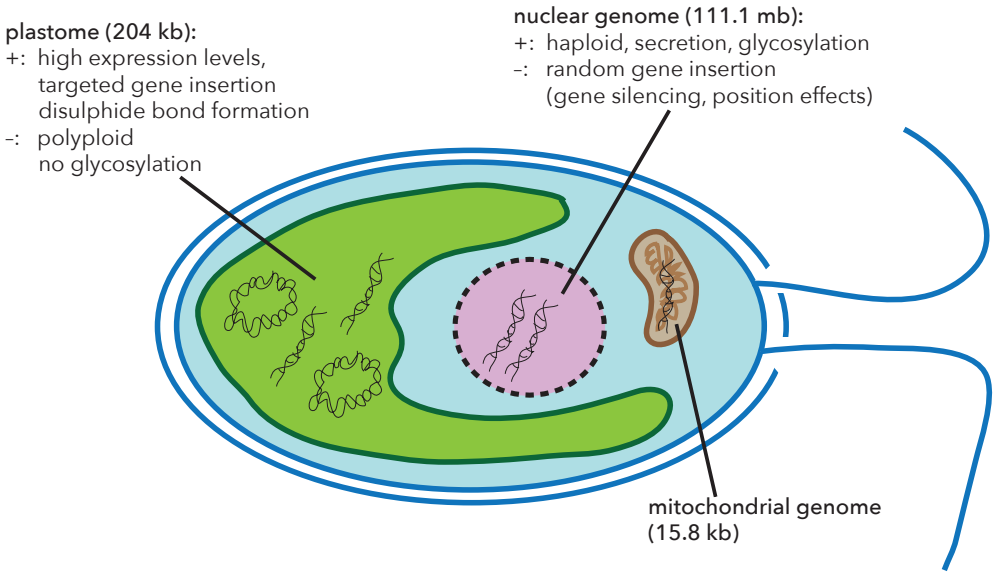


Fig. 1.1. Overview of genomes targeted for transgene insertion in *Chlamydomonas reinhardtii*. Major advantages (+) and disadvantages (–) for transgene expression from the respective genome are indicated in the figure.

The nuclear genome of *Chlamydomonas* consists of 17 haploid chromosomes and is approximately 111.1 Mb in size (Blaby *et al.*, 2014). A first annotated draft sequence had been published in 2007 (Merchant *et al.*, 2007) and major improvements have been implemented since then. The nuclear genome is relatively G+C rich with an approximate overall GC content of 64 % (Merchant *et al.*, 2007) and encodes a predicted number of 17 741 genes (Blaby *et al.*, 2014). Over the years classical genetic techniques have been established (Harris, 2009) and many mutants have been generated to study the physiology, biochemistry and reproduction of *Chlamydomonas* using a variety of mutagens and insertional mutagenesis methods (reviewed in more detail in e.g. (Jinkerson and Jonikas, 2015)). More targeted methods of editing the nuclear genome have also been reported for *C. reinhardtii*, but are, especially concerning the event of homologous recombination, not very efficient and would need further development to be of any use for heterologous gene insertion (reviewed in (Mussgnug, 2015)). The recently discovered CRISPR/Cas9 technology (Jinek *et al.*, 2012) might also be a new route to manipulate microalgae in the future. A first study applied this method to genetically modify *Chlamydomonas* (Jiang *et al.*, 2014) but considerable further improvements will be necessary to make this a viable option to transform this organism.

Homologous recombination seems to be a frequently occurring event in the nucleus of *Nannochloropsis* sp. (Kilian *et al.*, 2011) and can, therefore, be taken advantage of for targeted insertion of transgenes. However, in *Chlamydomonas reinhardtii* homologous recombination occurs much less frequently in the nuclear genome and is, thus, of no use for genetic engineering. Integration of heterologous genes into the nuclear genome occurs essentially at random and epigenetic gene silencing is a frequently observed issue (Cerutti *et al.*, 1997). Often, truncation of the cassette or genomic deletions, genomic rearrangements and others are thought to take place. How and why this occurs is not entirely known, but models of potential mechanisms exist (Zhang *et al.*, 2014).

Several ways of improving nuclear transformation efficiencies such as codon optimisation (Fuhrmann *et al.*, 1999) or the usage of introns in coding regions (Lumbreras *et al.*, 1998; Eichler-Stahlberg *et al.*, 2009) have been shown, but stable expression levels remain a difficult task and levels are low – Neupert et al. (2009) reported a relatively high level, 0.2 % of TSP, for fluorescent proteins using a new genetic screening method. More recently, approaches for a more modular and systematic design of expression systems from the nuclear genome have been developed for *C. reinhardtii* (Lauersen *et al.*, 2015) and further improvements using modular systems with a synthetic biology approach have been proposed (Scaife *et al.*, 2015).

The chloroplast genome has, in contrast, a very different nature. It is thought to have derived from a single event of endosymbiosis from a cyanobacterial ancestor (Kowallik, 1994). In *C. reinhardtii* the single plastid chromosome is approximately 204 kb in size with a G+C content of 34.6 % coding for approximately 99 expressed genes (Maul *et al.*, 2002). Most of these genes are encoding photosynthesis components or components of the translation machinery. The plastome also encodes *rbcL*, the large subunit of RuBisCO, probably the most abundant protein found on Earth. Approximately eighty copies of the plastome are found in *C. reinhardtii* (Boynton *et al.*, 1988) in multiple confirmations (both, circular and linear) (Maul *et al.*, 2002). This polyploidy depicts a challenge in itself for heterologous gene integration – homoplasmy is essential to ensure a stable genetic background of transformants.

Homologous recombination allows targeted gene integration in the chloroplast, which is a major advantage for genetic engineering (Bock, 2015). The levels of transgene expression in chloroplasts are much higher than from the nuclear genome, the

highest number reported to date is approximately 10 % TSP (Surzycki *et al.*, 2009), but usually levels achieved are not more than 5 % TSP (Potvin and Zhang, 2010).

Major factors affecting levels of protein accumulation in the chloroplast have been identified: codon optimisation (Franklin *et al.*, 2002; Wu *et al.*, 2011), protein degradation (the exposure to proteases) and genotypic modifications resulting from the transformation procedure (referred to as 'transformosomes') (Surzycki *et al.*, 2009). The toolkit has been developed remarkably over the last years, with various selectable markers, promoters and expression vectors now available (Purton *et al.*, 2013).

Depending on the nature of the heterologous gene one wants to express, the correct choice of integration site is crucial. Whereas the chloroplast offers higher expression levels and the amount of proteases acting on the heterologous protein is comparably low, the ability of PTM is rather limited: the chloroplast is capable of disulphide bond formation (Mayfield *et al.*, 2003), phosphorylation (Rasala and Mayfield, 2015) and N-acetylation of endogenous proteins has been observed frequently (Bienvenut *et al.*, 2011), but other PTMs such as *N*-glycosylation do not occur. Therefore, advances in expression of transgenes from the nuclear genome are very important. If the chloroplast capabilities are limited, there is a good chance that nuclear expression will offer a solution. For example, it is possible to secrete recombinant proteins to the culture medium (Rasala *et al.*, 2012; Lauersen *et al.*, 2013) or target them to various subcellular compartments (Hayashi and Shinozaki, 2012; Rasala *et al.*, 2014). Correct glycosylation of a recombinant protein, human erythropoietin, is expected when expressed from the nuclear genome of *C. reinhardtii* due to the intrinsic glycosylation properties of the organism. However, this could not be fully confirmed due to low protein yields and, therefore, missing experimental evidence (Eichler-Stahlberg *et al.*, 2009). A monoclonal antibody against Hepatitis B, produced in *P. tricornutum* was characterised biochemically demonstrating the diatom's glycosylation capabilities (Vanier *et al.*, 2015).

Understanding the properties of the specific recombinant protein targets is a first important step of choosing a suitable integration site. Microalgae offer various possibilities of expressing recombinant proteins and are considered of great potential for future applications. Rasala and Mayfield recently wrote a good review of what aspects should be taken into account when choosing the integration site (Rasala and Mayfield, 2015).

1.3.3. Promoters for nuclear and plastome expression in *C. reinhardtii*

The most commonly used promoters (5' untranslated region (UTR)) for nuclear expression are the *HSP70* and *HSP70/RBCS2* hybrid promoter (Schroda *et al.*, 2000). Another commonly used one is *PSAD*, which drives the expression of the photosystem I subunit PsaD (Fischer and Rochaix, 2001). Recently, a study used promoter trapping to identify further promoter sequences that are promising for nuclear expression systems (Vila *et al.*, 2012) and the *ARG7* promoter from argininosuccinate lyase was found to enable good expression levels (Specht *et al.*, 2015).

In the chloroplast several promoters have been used to drive transgene expression. Promoters from highly expressed endogenous algal genes turned out to result in the highest yields of recombinant protein (Walker *et al.*, 2005). Examples are the *rbcL* 5'UTR from the large subunit of RuBisCO (Blowers *et al.*, 1990; Franklin *et al.*, 2002), the ATP synthase subunit alpha promoter *atpA* (Blowers *et al.*, 1990; Mayfield *et al.*, 2003; Michelet *et al.*, 2011; Economou *et al.*, 2014), *psbA* (Michelet *et al.*, 2011; Gimpel and Mayfield, 2013), *psaA-exon1* (Michelet *et al.*, 2011; Young and Purton, 2014), the 16S rRNA promoter (Rasala *et al.*, 2011) or more recently *petA*, the promoter driving the expression of cytochrome f (Young and Purton, 2014).

The choice of promoter has a big impact on expression levels of a transgene and is itself regulated by external factors such as light in case of the *psbA* promoter for example. This has to be taken into account when choosing a 5'UTR for a heterologous gene construct. Re-engineering of the 5'UTR has shown to increase protein expression levels in the chloroplast of *C. reinhardtii* by bypassing feedback control mechanisms acting on the 5'UTR (Specht and Mayfield, 2013). Recently, a review was published that gives an extensive and far more complete overview of promoters used in *C. reinhardtii* for both chloroplast and nuclear expression (Mussgnug, 2015).

1.3.4. Selectable markers and reporter genes available for *C. reinhardtii*

Selectable markers and reporter genes are an important tool to detect if a heterologous gene has been integrated successfully. Whereas reporter genes such as fluorescent proteins or an antibiotic resistance cassette are used to select transformants

across all kingdoms, other markers are more specific for the respective organism transformed. Here, important markers and reporters described in both, the nuclear and the chloroplast genome of *C. reinhardtii* are introduced.

In *C. reinhardtii* positive transformation markers and reporter genes can be divided into the following categories (Mussgnug, 2015):

a) auxotrophy and complementation markers
b) antibiotic and herbicide resistance cassettes
c) reporter genes (colour or light emission detection)

Selectable markers for nuclear transformation in category a) are, for example, *ARG7* (Debuchy *et al.*, 1989) which complements arginine auxotrophy and *NIT1* (Kindle *et al.*, 1989) for restoring growth on nitrate as a nitrogen source. For chloroplast transformation, selectable markers of category a) are usually used for restoring photosynthetic capacities, for example *atpB* (Boynton *et al.*, 1988) or *psbH* (Cullen *et al.*, 2007). Category b) includes the *aadA* cassette from *Escherichia coli* lending resistance to streptomycin and spectinomycin. This marker has been used in both, the chloroplast genome (Goldschmidt-Clermont, 1991) and the nuclear genome (Cerutti *et al.*, 1997). Other antibiotic resistance cassettes used for nuclear transformation include a *ble* cassette for resistance to bleomycin, phleomycin and zeocin/zeomycin (Stevens *et al.*, 1996), resistance to chloramphenicol via integration of the *cat* gene into the nuclear genome (Tang *et al.*, 1995) or resistance to hygromycin via expression of the *hpt* gene from *E. coli* (Butanaev, 1994; Kumar *et al.*, 2004).

A reporter gene used for detecting 'colour' is for example β-Glucuronidase (*uidA* gene from *E. coli*) (Kumar *et al.*, 2004) expressed from the nuclear genome. A variety of fluorescent proteins have been expressed in *C. reinhardtii* in various compartments including GFP (and modified versions thereof) (Fuhrmann *et al.*, 1999; Franklin *et al.*, 2002; Kumar *et al.*, 2004; Neupert *et al.*, 2009) and other fluorescent proteins (Rasala *et al.*, 2013; Lauersen *et al.*, 2015) and also chemiluminescent proteins (Fuhrmann *et al.*, 2004; Shao and Bock, 2008). A more detailed overview of selectable markers and reporter genes is given in (Gangl *et al.*, 2015a) and Supplementary Material Table S2 in (Mussgnug, 2015).

1.3.5. The chloroplast transformation vectors pASapI and pSRSapI

In this section the details of the chloroplast transformation vectors used to make the transformants described in this thesis will be introduced. The constructs were made in the lab of Saul Purton (University College, London, UK). PASapI and pSRSapI have been developed for chloroplast transformation of *C. reinhardtii* using the glass bead method (Economou *et al.*, 2014). The transformation method uses a strain, previously generated by genetic engineering, named TN72 (cw15, *psbH::aadA,* mt+). Upon transformation, the *aadA* cassette disrupting *psbH* in TN72 is exchanged with a functional copy of *psbH* and simultaneously the heterologous gene gets recombined into the chloroplast genome. This allows the generation of marker free transformants that essentially only contain the gene of interest (GOI) of foreign DNA.

Positive transformants are selected via growth on minimal medium because by integrating a functional copy of the *psbH* gene, which encodes a subunit of photosystem II, the photosynthetic capacity of the strain is restored. TN72 has to rely on an organic carbon source for growth because of the disrupted *psbH* gene whereas the then generated transformants can grow photoautotrophically. Selection for autotrophic growth is a strong selection pressure, thus homoplasmic transformants are relatively easy to obtain with this method.

PASapI (Economou *et al.*, 2014) and pSRSapI (Young and Purton, 2014) are almost identical vectors: they integrate into the same site in the chloroplast genome and for both, TN72 is used as a recipient strain. The heterologous gene can be cloned into the vectors rather easily with a classical restriction digest and subsequent ligation of the insert into the vector using the restriction enzymes *SapI* (*LguI*) and *SphI* (*PaeI*). The difference between pASapI and pSRSapI is the 5'UTR. In pASapI an *atpA* promoter drives expression of the heterologous gene whereas in pSRSapI a *psaA* promoter is used. The *psaA* promoter should result in approximately a 2-fold increase of expression compared to the *atpA* promoter (Saul Purton, personal communication). An overview of the vector elements of the vectors is given in Fig. 1.2 for pASapI (A) and pSRSapI (B). Fig.1.3 schematically represents the procedure for obtaining *C. reinhardtii* chloroplast transformants using the pASapI or pSRSapI vector.

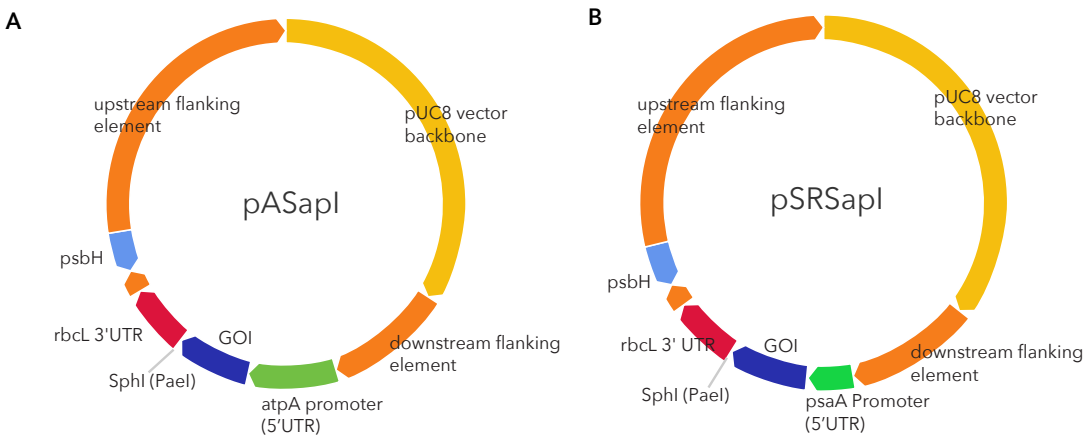


Fig. 1.2. pASapI and pSRSapI vector elements. Elements of the pASapI (Economou *et al.*, 2014) (**A**) and pSRSapI (Young & Purton, 2014) (**B**) vectors for chloroplast transformation of *C. reinhardtii* are shown. An example GOI (gene of interest) of 500 bp is cloned into the vector. Upon integration of the GOI, the restriction site *SapI (LguI)* gets destroyed and is therefore not shown in the schematic drawing. The size of the vector elements is in proportion with their length in base pairs. Vector diagrams were assembled using the software Gene Designer (DNA 2.0) according to information obtained from Saul Purton (UCL, UK).

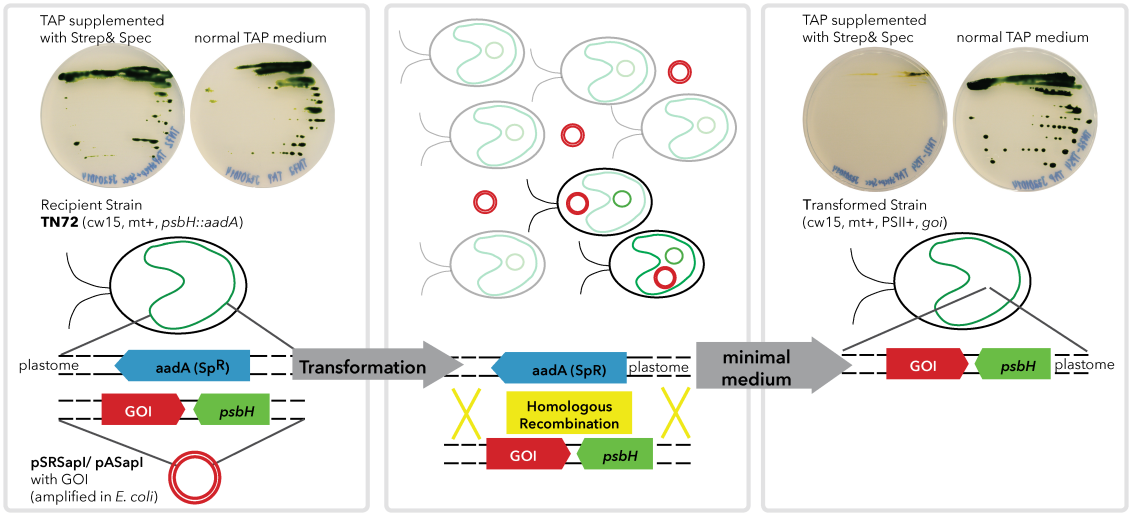


Fig. 1.3. Overview of *C. reinhardtii* chloroplast transformation procedure using pASapI/pSRSapI. An overview of the chloroplast transformation and transformant selection procedure using the method described in Economou *et al.* (2014) is shown. Details are explained in section 1.3.4. Figure adopted from Supplementary Material ESM1 in (Zedler *et al.*, 2015).

1.3.6. Future challenges in genetically modifying microalgae

One of the major challenges associated with more complex genetic engineering in the future will be to coordinate the expression of several genes. In the case of metabolic pathway engineering, the expression levels might also have to be fine-tuned to allow for effective product synthesis. Expressing several heterologous genes, simultaneously, in eukaryotic microalgae is to date not established. Engineering of complex operons with polycistronic constructs, which is possible in cyanobacteria, is not advanced and might not be possible due to a different organisation of the genetic information (this is especially the case for nuclear expression systems).

Theoretically, the expression of multiple genes in the chloroplast of e.g. *C. reinhardtii* should be possible by inserting transgenic operons (Purton *et al.*, 2013), but this method has, to date, not been used with a lot of success and only very few studies report the expression of several genes (Su *et al.* 2005, Chaogang *et al.*, 2010). Methods for multiple gene expression in the chloroplast of *C. reinhardtii* (Noor-Mohammadi *et al.*, 2012) and also in the nuclear genome (Noor-Mohammadi *et al.*, 2014; Rasala *et al.*, 2014) have been proposed. However, all these studies did not engineer true biochemical pathways but only used reporter and selectable marker genes. Therefore, this is not comparable with actual metabolic pathways that will have a considerable impact on the cellular metabolism. In another study, editing the chloroplast genome, simultaneously at several sites, is proposed by assembling a full chloroplast genome exogenously and subsequent transformation into the chloroplast (O'Neill *et al.*, 2012). The method has many problems with DNA stability, for example due to the repetitive nature of the chloroplast genome, and, if this is to be of use for genetic engineering, further improvements are needed. Another study shows the successful use of a FMDV 2A self-cleavage peptide for expressing two genes as one construct from the nuclear genome in *C. reinhardtii* (Rasala *et al.*, 2012). However, this self-cleavage site is unfortunately not recognised in a prokaryotic environment, as found in the chloroplast of microalgae, and is, therefore, of no use for expressing several genes from the chloroplast genome.

Other challenges have been addressed in more detail in previous studies and remarkable progress was already made, such as improved protein expression levels and identification of major factors impacting protein expression levels (Manuell *et al.*, 2007;

Surzycki *et al.*, 2009). Recently, selenocystamine was reported to increase accumulation of recombinant proteins containing disulphide bonds in the chloroplast, probably by assisting with disulphide bond formation (folding) (Ferreira-Camargo *et al.*, 2015). The synthesis of toxic substrates (Tran *et al.*, 2013a; Tran *et al.*, 2013b; Young and Purton, 2015) or challenges such as secretion of products into the culture medium (Rasala *et al.*, 2012; Lauersen *et al.*, 2013) have also been addressed previously, but there is still room for improvement.

1.4. Genetic engineering of microalgae for biotechnological applications

In Section 1.3 important tools and techniques for genetically modifying microalgae have been introduced. The following section will focus on the products that have been made in microalgae for biotechnological applications using those tools. The production of recombinant proteins will be discussed separately from high-value compounds for *C. reinhardtii* and an overview of high-value products (including recombinant proteins) in other eukaryotic microalgae will be given.

1.4.1 Expression of recombinant proteins in *C. reinhardtii*

The expression of recombinant proteins in microalgae has advanced significantly over the last 10 years, especially for *C. reinhardtii*. To date, over 50 proteins have been expressed successfully in the chloroplast of *Chlamydomonas* (Purton *et al.*, 2013). First reports of recombinantly expressed proteins occurred shortly after transformation protocols had been developed. Later on, in 1991, the functional expression of a heterologous gene, *aadA* from *E. coli* was shown in *C. reinhardtii* by conferring resistance to the transformants (Goldschmidt-Clermont, 1991). The first report of a therapeutic protein of interest for biotechnological applications expressed in microalgae only occurred more than ten years later (Mayfield *et al.*, 2003) – the transformant was made by particle bombardment and expressed an HSV8-lsc single-chain, monoclonal human antibody driven by *atpA* and *rbcL* 5'UTRs. After this, the

number increased rapidly. Most of the recombinant proteins produced in *Chlamydomonas* for biotechnological applications can be classified in the following categories:

a) Antibodies and immunotoxins, vaccines
b) Other therapeutic proteins (including hormones)
c) Enzymes

After the first scFv (single chain Fragment variable) antibody was produced, Tran *et al.* showed that it is also possible to assemble a full-length antibody in the chloroplast of *C. reinhardtii*. The human IgG1 antibody against anthrax was shown to have very similar binding activities to an antibody produced in mammalian cell culture (Tran *et al.*, 2009). The chloroplast of *C. reinhardtii* has also been used for expressing immunotoxins. Immunotoxins are conjugates of an antibody fused to a eukaryotic toxin domain used in cancer therapeutics. The antibody domain recognises the targeted cell and the toxin then initiates killing of the cancer cell (Alewine *et al.*, 2015). The production of these molecules is not possible in eukaryotic cells (yeast, Chinese hamster ovary (CHO) cells, insect cells) due to toxicity. They can be produced in *E. coli,* which is, however, not capable of all necessary PTMs. *C. reinhardtii* has been shown to be capable of assembling functional immunotoxins, both monomeric and dimeric, using the PE40 domain of exotoxin A from *Pseudomonas aeruginosa* (Tran et al., 2013a) and also a eukaryotic ribosome inactivating protein, gelonin (Tran *et al.*, 2013b), as the toxin unit. The microalgal chloroplast offers a unique opportunity to produce these toxins due to the prokaryotic translation machinery coupled with eukaryotic capabilities in regard to PTMs and protein stability. Several patent applications for the production of these immunotoxins in microalgae have been filed by the authors, but only the European patent was granted recently (Mayfield and Tran, 2014). The group of S.P. Mayfield recently also produced camelid antibody fragments that act as an antitoxin against the botulinum neurotoxin in the chloroplast of *C. reinhardtii* (Barrera *et al.*, 2015).

The most reported therapeutic proteins produced in *C. reinhardtii* are various kinds of vaccines. This is possibly due to the potential of oral vaccination using lyophilised harvested biomass (if the microalga used for production has GRAS status) (Rosales-Mendoza, 2013). Oral administration of vaccines allows to target mucosal and

systemic immunity simultaneously, and vaccines produced in algae pose a lower risk of pathogen contamination when compared to vaccines produced in mammalian cells (Specht and Mayfield, 2014). Very early on, in recombinant protein production systems developed in microalgae, a virus VP1 protein (fused with a Cholera toxin subunit) against Foot-and-Mouth disease virus was produced in the chloroplast accounting to 3 % of TSP in the chloroplast, however, the generated strains were not homoplasmic (Sun *et al.*, 2003). The first demonstration of an oral vaccine produced in algae was a vaccine protecting mice from *Staphylococcus aureus* infections upon oral administration (Dreesen *et al.*, 2010). Subsequently vaccines against the classical swine fever virus (even though oral application to mice did not result in a response, only when applied subcutaneously) (He *et al.*, 2007), an antigen against hGAD65 for diabetes type I treatment (Wang *et al.*, 2008) and a white spot syndrome virus VP28 protein (Surzycki *et al.*, 2009) were reported. Further vaccines expressed in *C. reinhardtii* include an E7 vaccine against the human papilloma virus (Demurtas *et al.*, 2013) and a vaccine against angiotensin II for hypertension treatment (although no immunological assays showing activity are given) (Soria-Guerra *et al.*, 2014). There is also evidence from several studies that vaccines for malaria treatment can be made in *C. reinhardtii* (Dauvillée *et al.*, 2010; Gregory *et al.*, 2012; Gregory *et al.*, 2013; Jones *et al.*, 2013) and again, patents for the production of malaria transmission blocking vaccines have been filed by authors of the studies, but have not been granted to date (patent applications: US 20140219971, WO 2012170125, WO 2012170125).

Other therapeutic proteins expressed in *C. reinhardtii* include human hormones such as proinsulin (at very low levels only detectable by immunoprecipitation of 50 mL cultures) (Rasala *et al.*, 2010), vascular endothelial growth factor (Rasala *et al.*, 2010) and erythropoietin (Eichler-Stahlberg *et al.*, 2009). One of the first attempts at co-expression of two genes in one construct from the chloroplast genome was made in 2005; two subunits of allophycocyanin, *apcA* and *apcB* were expressed at approximately 25 µg per mg of TSP. Potential inhibition of S-180 carcinoma cells in mice by this recombinant allophycocyanin is indicated, but no data are presented (Su *et al.*, 2005). Yang *et al.* produced a non-codon optimised TRAIL (tumor necrosis factor-related apoptosis inducing ligand) protein that selectively causes apoptosis in tumor and virus-infected cells (Yang *et al.*, 2006). In a study replacing the coding region of *psbA* in the chloroplast directly with the coding sequence of bovine mammary-associated serum amyloid (M-SAA), high levels of up to 5 % TSP of the recombinant protein were

reported. M-SAA is a protein that is able to stimulate mucin production in epithelial cells of the human gut for enteric bacterial and viral infection prophylaxis (Manuell *et al.*, 2007), the expression system has also been patented by the authors (Mayfield, 2010; Mayfield, 2015). Purton *et al.* showed that endolysins, an alternative to traditional antibiotics, can be produced in *C. reinhardtii* (Taunt (2013), Saul Purton *et al.*, unpublished, patent application 704751).

In line with using microalgae as functional foods, a study examined the feasibility of expressing milk-derived bioactive peptides in *C. reinhardtii* with antihypertensive, opioid, antimicrobial and hypocholesterolemic activities. The study showed that the chimeric proteins could be expressed in microalgae, but the levels were variable (Campos-Quevedo *et al.*, 2013).

Enzymes expressed in *C. reinhardtii* include various classes. Yoon *et al.* produced a phytase from *E. coli* that increased phytate phosphorus utilisation as a feed additive when fed to boiler chickens (although chicken tests were conducted with only n=3 chickens and supplementing the chicken's diet with lyophilised WT *C. reinhardtii* (strain CC-125) also resulted in decreased phytate levels) (Yoon *et al.*, 2011). Other enzymes expressed include ADH1, alcohol hydrogenase, which resulted in ethanol production in the transformed *C. reinhardtii* strains (Chen and Melis, 2013) and an industrially relevant enzyme, xylanase (produced in the cytosol and secreted to the medium) (Rasala *et al.*, 2012).

An enzyme expressed in the chloroplast by Fukusaki *et al.* is of major interest for this thesis as it encodes a GGPP synthase that generates GGPP, the universal precursor of diterpenes. A *gds* gene from a thermophilic archaeon was introduced with the aim to engineer the secondary metabolism of *C. reinhardtii*. The enzyme, GGPP synthase, was detected but differences between WT and the transformant were only found at higher temperatures (possibly due to the enzyme originating from a thermophilic organism) which can be a problem for the normal cellular metabolism of *C. reinhardtii* (Fukusaki *et al.*, 2003).

This section only highlighted major achievements in recombinant protein production, several reviews provide a deeper insight into what proteins have already been made in algae (e.g. (Mayfield *et al.*, 2007; Specht *et al.*, 2010; Rosales-Mendoza *et al.*, 2012; Almaraz-Delgado *et al.*, 2014; Specht and Mayfield, 2014; Rasala and Mayfield, 2015)).

1.4.2. Other high-value compounds produced in *C. reinhardtii*

Studies reporting the production of high-value compounds (by genetic engineering) other than recombinant proteins in *C. reinhardtii* are, to date, very rare. Expression of the β-carotene hydroxylase from *Haematococcus pluvialis* in *C. reinhardtii* resulted in an elevated carotenoid content compared to the WT when exposed to high light (Tan *et al.*, 2007). In 2010, Chaogang *et al.* showed that PHB, a biodegradable polyester, can be produced and accumulated in *C. reinhardtii*. PHB production costs are still very high and microalgae could provide an alternative, cheaper, production system (Chaogang *et al.*, 2010). One study reports the production of xylitol, an artificial sweetener, in the chloroplast by expression of a xylose reductase from *Neurospora crassa* and feeding of xylose into the culture medium (Pourmir *et al.*, 2013). However, the sugar can be produced easily in yeast and *E. coli* and NAD(P)H availability seems to be limited in the chloroplast and therefore also limiting the product formation.

1.4.3. High-value compounds produced in other microalgae

Most reports of genetic modification in microalgae are in *C. reinhardtii*. In more recent years, increasingly other microalgae have been subject to genetic modification studies. Model organisms studied are mainly species that have already been used in an industrial setting and that are known to be scalable. In most cases metabolic engineering studies focus on engineering for improved lipid synthesis towards biofuel production in microalgae (e.g. in *P. tricornutum* (Hamilton et al., 2015), *Nannochloropsis oceanica* (Kaye *et al.*, 2015), *Schizochytrium* sp. (Yan *et al.*, 2013) and *Fistulifera solaris* (Muto *et al.*, 2015)). Recently several reviews have been published summarising developments in lipid synthesis engineering in microalgae (De Bhowmick *et al.*, 2015; Gimpel *et al.*, 2015a; Levering *et al.*, 2015).

In *Phaeodactylum tricornutum* a monoclonal antibody against Hepatitis B was produced. The antibody was expressed at 8.7 % of TSP from the nuclear genome in the ER (Hempel *et al.*, 2011b) and later on it was shown that the IgG antibody can also be secreted to the medium (Hempel and Maier, 2012). In a second follow-up study, the

quality of the produced antibodies was characterised biochemically and found to be of high and consistent quality (Vanier *et al.*, 2015). Similarly, HBsAg has been produced in *Dunaliella salina* (Geng *et al.*, 2003) and more recently, haemagglutinin has been produced in the microalga *Schizochytrium* sp. (Bayne *et al.*, 2013).

The expression of hormones has been reported for other eukaryotic microalgae. Human growth hormone has been expressed in *Chlorella* (Hawkins and Nakamura, 1999). A flounder growth hormone produced in *Chlorella ellipsoidea* increased flounder fry growth by 25 % when fed with zooplankton that had incorporated the transgenic *Chlorella* (Kim *et al.*, 2002).

As with what has been done in *C. reinhardtii* by Chaogang *et al.* (2010), a study also investigated PHB biosynthesis in *P. tricornutum* (Hempel *et al.*, 2011a). The synthesis of engineered high-value compounds other than naturally abundant high-value compounds in microalgae (see section 1.2.2.2) is still rare.

1.5. Utilising the microalgal metabolism for biotechnological applications

This section will highlight aspects of the microalgal metabolism that have potential for future biotechnological applications. The focus will be on traits that have been investigated and proposed for genetic engineering targets as part of this thesis.

1.5.1. Isoprenoid synthesis in microalgae

Isoprenoids are hydrocarbons of enormous chemical and structural diversity found in all living organisms. They serve, for example, as pigments, hormones, defensive agents, constituents of membranes and photoprotective agents (Sacchettini and Poulter, 1997). The greatest diversity is however found in plants where isoprenoids are synthesised as secondary metabolites (Englund *et al.*, 2015). Isoprenoids are also referred to as terpenes or terpenoids, names that are used interchangeably (Lohr *et al.*, 2012).

There are two known pathways in nature for isoprenoid synthesis: the mevalonate (MVA) pathway (HMG~CoA reductase pathway) and the methylerythritol phopshate (MEP) (the MVA-independent) pathway. The MVA pathway forms IPP (isopentenyl diphosphate, the active precursor molecule of all isoprenoids) from acetyl~CoA, whereas IPP is synthesised from pyruvate and glyceraldehyde-3-phosphate (GA3P) via the MEP pathway.

The MVA pathway was long thought to be the only route for isoprenoid synthesis as the MEP pathway was only fully elucidated and accepted in more recent years (Eisenreich *et al.*, 2004). In both cases, the three major stages for the biosynthesis of isoprenoids are as follows (Lohr *et al.*, 2012):

1. Formation of active isoprene (IPP or DMAPP (dimethylallyl diphosphate))
2. Linear condensation of C5 units to polyprenyl diphosphates (variable chain length)
3. Further modification of the polyprenyl diphosphates

The general consensus is that the MVA and the MEP pathways operate in different cellular compartments. For example in streptophytes (including land plants), the MVA pathway operates in the cytosol whereas the MEP pathway operates in the plastids, but more recently active metabolite trafficking and cross regulation was uncovered from several studies (reviewed in (Lohr *et al.*, 2012)). In many oxygenic phototrophs, including chlorophytes (green algae), the MVA pathway is absent. For example, in *C. reinhardtii* no evidence for the pathway was found in its genome sequence or in labelling experiments (Disch *et al.*, 1998), whereas in other microalgae (especially with secondary plastids) such as the diatom *P. tricornutum,* both, the MVA and the MEP pathway seem to operate (Cvejić and Rohmer, 2000). A model of how isoprenoid synthesis metabolites are compartmentalised in eukaryotic green algae, such as *C. reinhardtii*, where only the MEP pathway is present, is shown in Fig 1.4.

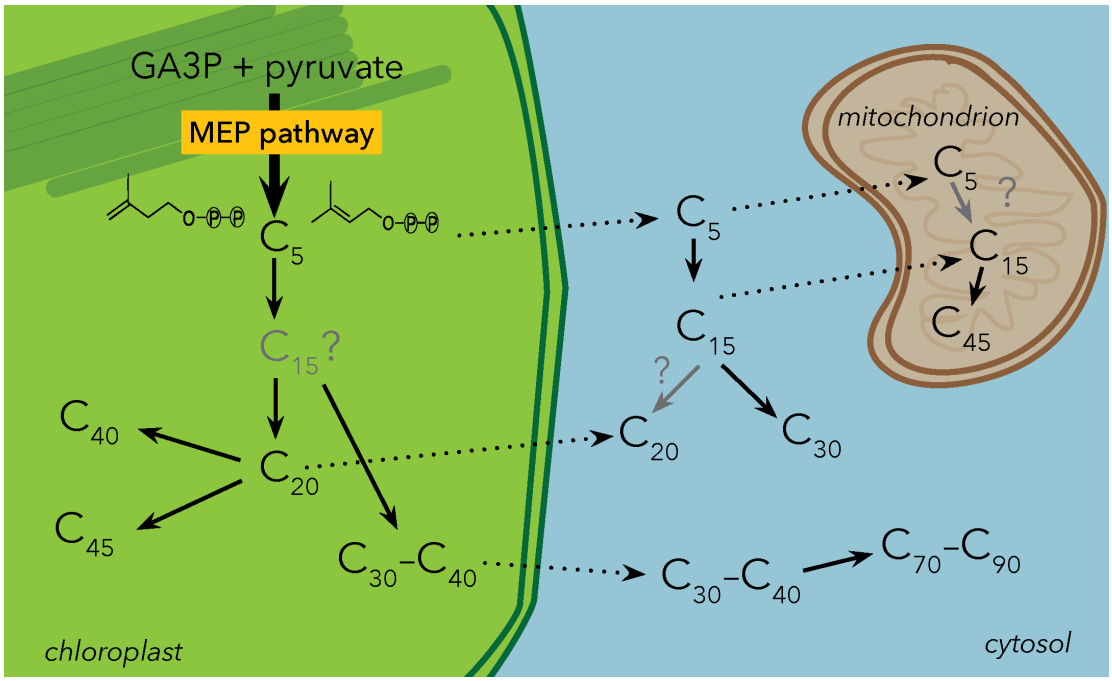


Fig. 1.4. Compartmentation of isoprenoids and trafficking of precursors in eukaryotic microalgae where only the MEP pathway operates. This is a hypothetical compartmentation based on biochemical and *in silico* data. Dotted arrows show potential transport of isoprenoid precursor units between compartments across membranes. The length of the isoprenoid precursor molecule is indicated (C_X). GA3P: glyceraldehyde-3-phosphate. Ⓟ: phosphate group. Grey colour and question marks indicate putative catalysis (enzymes might be missing). Figure was adopted and modified from Fig. 4A in (Lohr *et al.*, 2012).

The smallest isoprenoid is the C5 molecule isoprene. Isoprenoids with a longer chain length are all assembled with the 5-carbon unit of IPP (DMAPP) and named according to their chain length following the "isoprene rule" (Ruzicka, 1953): monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20) and so on. Further diversity originates from different degrees of saturation and rearrangements of the structure (linearisation, cyclisation, isomerisation and branching) (Davies *et al.*, 2015).

Isoprenoids are of major interest for the synthesis of both commodity and high-value compound production in photosynthetic hosts. However, genetic engineering for establishing a photosynthetic isoprenoid production platform is still in its early days. Major advances in redirection of carbon flux and complex pathway engineering will be necessary (Davies *et al.*, 2015). *Synechocystis* sp. PCC 6803 has been studied as a cyanobacterial model system for isoprene production (Lindberg *et al.*, 2010), β-carophyllene (a sesquiterpene) (Reinsvold *et al.*, 2011), the monoterpene β-pyllandrene (Bentley *et al.*, 2013) and manoyl oxide (a diterpene) (Englund *et al.*, 2015) synthesis.

However, to date no studies of recombinant synthesis of terpenes in eukaryotic microalgae have been reported, but their potential has been indicated several times (Lohr *et al.*, 2012; Work *et al.*, 2013; Davies *et al.*, 2015).

1.5.2. Engineered, light-driven isoprenoid (terpene) synthesis in microalgae and cyanobacteria

In this section a novel approach of genetic engineering to couple reducing power from photosynthesis to high-value compound production is introduced. This field of research has been pioneered by researchers from the University of Copenhagen and recently great progress has been made. The general idea behind this is to scavenge reducing power from the photosynthetic electron transport chain (PET). This is possible because energy from light is often harvested in excess compared to what can be further metabolised which essentially allows "light-driven *in vivo* product synthesis" (Jensen *et al.*, 2012). The scavenged reducing power can then be used to facilitate catalysis of high-value compounds that are of interest for synthetic biology (Lassen *et al.*, 2014b). There are several groups of secondary metabolites produced by plants that are of interest for production in microalgal photosynthetic hosts, including isoprenoids, alkaloids and flavonoids (Marienhagen and Bott, 2013).

Terpenes from plants are used in a variety of industries – as pharmaceuticals, flavours, fragrances, food supplements (vitamins and sweeteners), pesticides and even as feedstock for many industrial materials (Bohlmann and Keeling, 2008). Typically these compounds are produced at only very low quantities in the natural host (plants) and are often too complex for chemical synthesis, therefore, microorganisms are considered an attractive alternative production platform (Marienhagen and Bott, 2013). Terpene biosynthesis typically involves two major classes of enzymes: terpene synthases that form the scaffold of the molecule and cytochromes P450 (P450s), a large class of monooxygenases, that functionalise the scaffolds by oxidation (Pateraki *et al.*, 2015). Most of the P450s involved in terpene synthesis in plants are class II P450s that are membrane bound enzymes located in the ER. P450 activity is then typically limited by NAD(P)H availability. Therefore, relocation of those P450s to the thylakoid membranes where reducing power can be provided by PET could allow increased

activity and consequently higher product yields (Lassen *et al.*, 2014b). The relocation of P450s has been described in several proof of concept studies: first, by transient expression in tobacco and targeting of the P450s to the chloroplast (Nielsen *et al.*, 2013). Later, in the cyanobacterium *Synechococcus* sp. PCC 7002 the soluble subunit of a P450 (CYP79A1) was anchored to a PSI subunit (PsaM) for targeting to the thylakoids allowing catalysis *in vitro* and *in vivo* (Lassen *et al.*, 2014a). More recently it was shown that the whole pathway for dhurrin synthesis can be engineered into *Synechocystis* sp. PCC 6803 involving relocated P450s (Wlodarczyk *et al.*, 2016).

Theoretically, the relocation of P450s as shown in other model systems could also be used for terpene synthesis in eukaryotic microalgae. A model of how recombinant expression of terpene synthases and P450s in the *C. reinhardtii* chloroplast could facilitate *in vivo* terpene synthesis integrated into the algal metabolism is shown in Fig. 1.5.

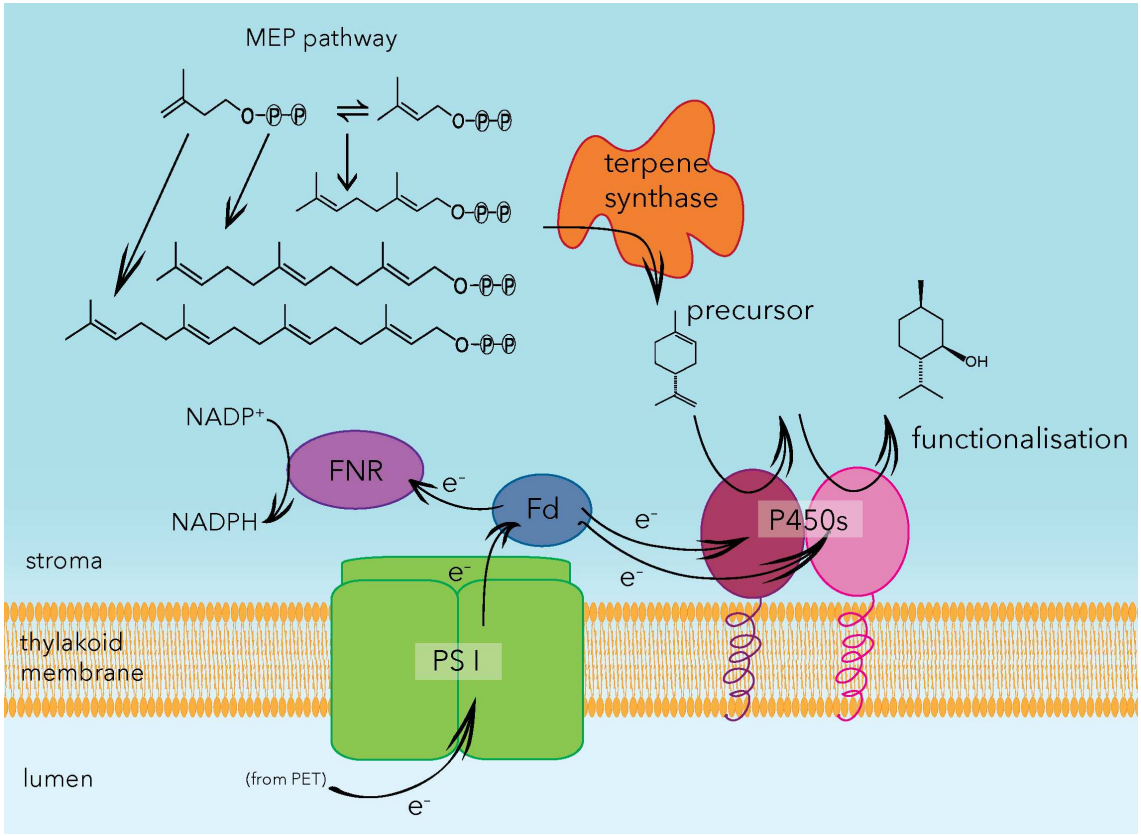


Fig. 1.5. Model of potential light-driven terpene production in the chloroplast of *C. reinhardtii.* A hypothetical model outlining terpene production in the *C. reinhardtii* chloroplast is depicted. Terpene precursor molecules are synthesised via the MEP pathway and a precursor terpene is synthesised by terpene synthases that gets functionalised by cytochromes P450 (P450s) that were relocated and inserted in the thylakoid membranes. Ferredoxin (Fd) feeds electrons (e^-) from the photosynthetic electron transport chain (PET) to drive the P450s involved in terpene synthesis and the Ferredoxin $NADP^+$ reductase (FNR) at the same time.

1.5.3. Protein targeting in the algal chloroplast and the thylakoids

Protein targeting is a highly sophisticated and complex process found in all living organisms. The understanding and utilisation of these trafficking mechanisms are crucial for proteins to be targeted and produced in the algal chloroplast. Chapter 4 introduces a novel approach of utilising the thylakoid lumen for recombinant protein expression in algae. Here, details of targeting mechanisms across the chloroplast envelope membranes and into (and across) the thylakoid membranes will be introduced.

1.5.3.1. Targeting of nuclear encoded proteins into chloroplasts

The primary plastids found in members of the Viridiplantae, including eukaryotic green microalgae (Chlorophyta) and higher plants (Streptophyta) (Leliaert *et al.*, 2012) are thought to have originated from a single endosymbiotic event and to be of cyanobacterial ancestry. Plastid import mechanisms are therefore considered to be rather similar, although, after an estimated divergence of 725 – 1200 million years (Becker and Marin, 2009), differences are not surprising. Most of the proteins operating in the chloroplast are nuclear encoded and thus need to be targeted, post translation, to the chloroplast by means of transit peptides. Import of proteins into primary plastids means that the substrate has to be translocated over the outer envelope membrane and the inner envelope membrane that are separated by a soluble intermembrane space. Two major complexes, TOC and TIC, located in the outer and inner envelope membranes facilitate this process (Jarvis and Soll, 2001). A bioinformatics analysis of the TOC and TIC complexes found that the composition of those complexes in *C. reinhardtii* is very similar to *Arabidopsis thaliana* (Kalanon and McFadden, 2008). A major difference is the nature of the transit peptides – in general, chloroplast transit peptides of green microalgae often share similarities with mitochondrial presequences of higher plants (Franzén *et al.*, 1990). This is a major constraint for subcellular localisation prediction using programmes such as TargetP (Emanuelsson *et al.*, 2000) or ChloroP (Emanuelsson *et al.*, 1999) designed for higher plants. Efforts to address these limitations were already made and software for predicting protein targeting in green algae has been developed (PredAlgo, (Tardif *et al.*, 2012)). Upon translocation into the stroma of the plastid, the signal peptide is typically cleaved off by the stroma processing peptidase (Richter and Lamppa, 2003).

Many nuclear encoded proteins need to be not only targeted to the chloroplast stroma, but across or into another membrane - the thylakoid membrane. This means than an additional signal peptide is needed in the preprotein, for which reason these proteins have a bipartite N-terminal signal sequence (Ko and Cashmore, 1989). A schematic overview of the journey from a nuclear encoded protein into the thylakoid lumen is shown in Fig. 1.6. It is crucial to gain a deep understanding of the translocation and targeting mechanisms in algae to be able to precisely target proteins to subcellular

compartments for designing engineered metabolic pathways and for developing microalgae as cell factories.

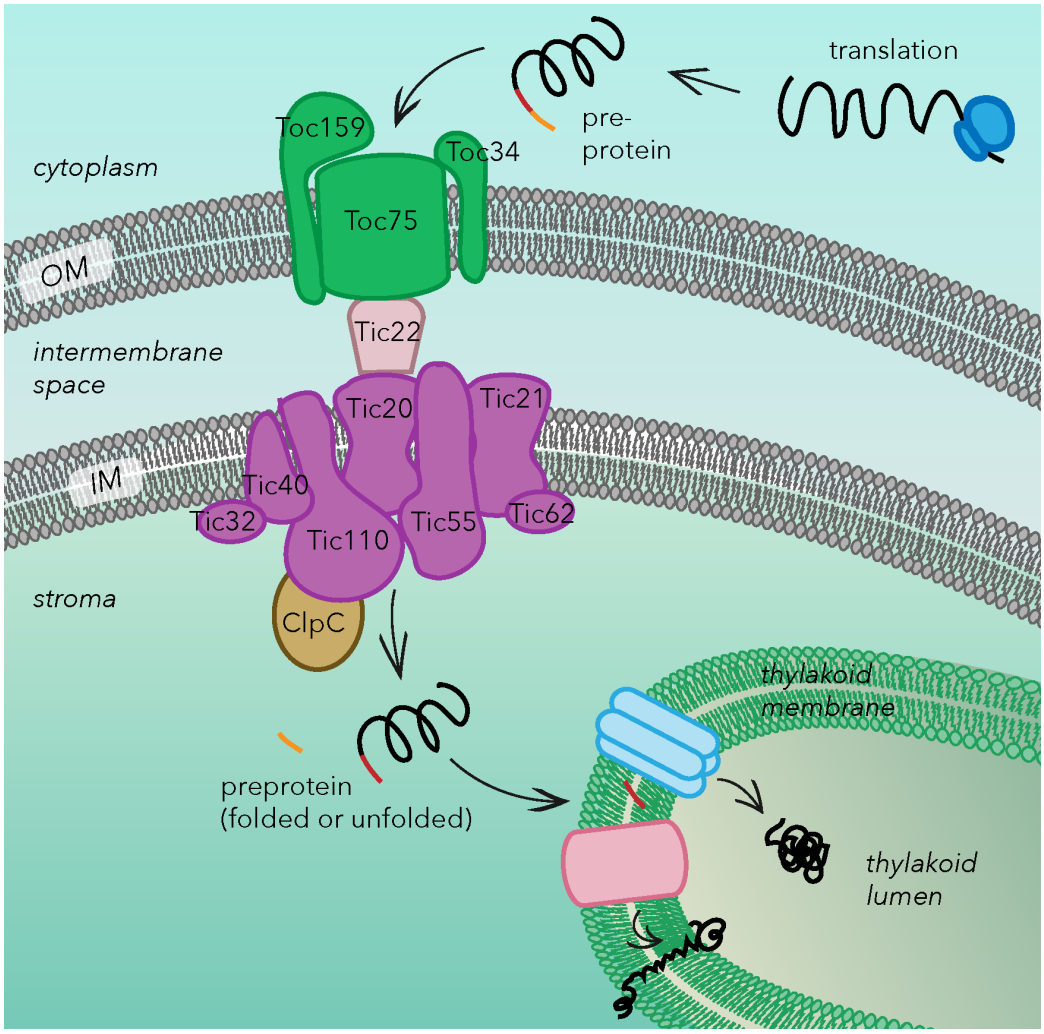


Fig. 1.6. Import of nuclear encoded protein with bipartite N-terminal signal sequence in *C. reinhardtii*. A model of a nuclear encoded protein that is translated in the cytoplasm and then imported into the thylakoids is shown. The protein is translocated into the stroma via the TOC and TIC complexes in the outer envelope membrane (OM) and the inner envelope membrane (IM) of the chloroplast. The TOC membrane complex consists of the subunits Toc159, Toc75 and Toc34. The TIC complex is formed by the following subunits: Tic20, Tic21, Tic 22, Tic 32, Tic40, Tic55, Tic110, Tic62. Additionally the stromal chaperone ClpC binds to the Tic complex assisting translocation of the target proteins. Subunits of the complexes are shown in the figure. In the stroma the first part of the signal sequence is removed. A second part of the N-terminal signal sequence then facilitates translocation into or across the thylakoid membrane, where the second part of the signal sequence is removed. The folding state of the protein in which it is transported depends on the translocation mechanism (see Fig. 1.7.). The figure is based on information from (Kalanon and McFadden, 2008) and (Teixeira and Glaser, 2013).

1.5.3.2. Insertion into the thylakoid membrane and targeting to the lumen

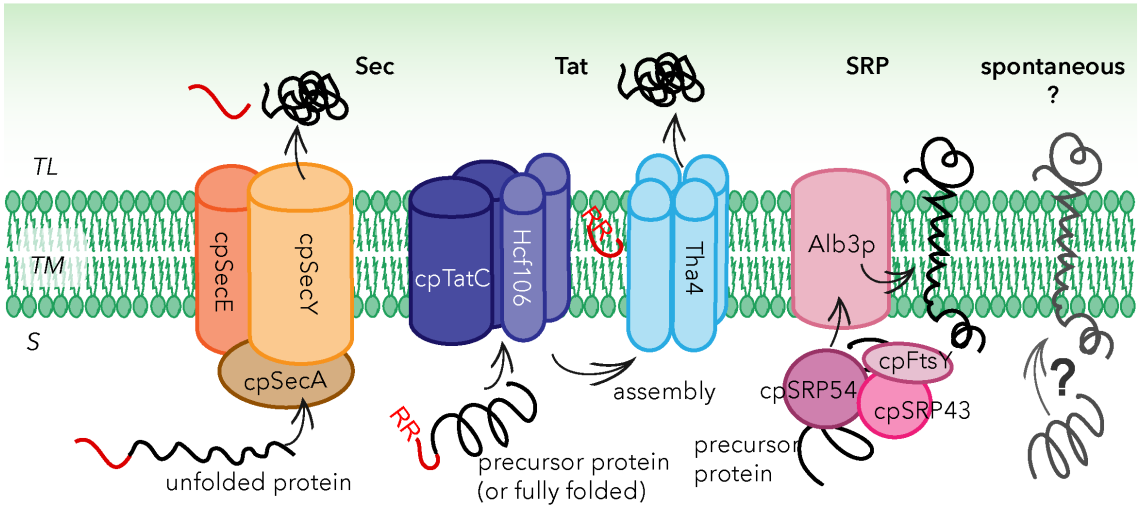


Fig. 1.7. Protein translocation mechanisms in chloroplast thylakoids. The major components of the respective translocation machinery (Sec, Tat, SRP) are shown and the nature of the substrate transported (folded / unfolded) is indicated. TL: thylakoid lumen; TM: thylakoid membrane; S: stroma. Figure was modified from original (Fig. 3) compiled for a review about protein translocation and thylakoid biogenesis in cyanobacteria (Frain *et al.*, 2016).

Generally, proteins are translocated to the thylakoid lumen via either the Secretory (Sec) translocation machinery or the Twin-arginine translocation (Tat) system (Albiniak *et al.*, 2012). Insertion of proteins into the thylakoids is mainly mediated by the signal recognition particle (SRP) pathway, but additionally a (debatable) second spontaneous pathway may be operating in thylakoids (Robinson *et al.*, 2001; Aldridge *et al.*, 2009). Fig. 1.7 gives an overview of the multiple pathways for protein targeting in the thylakoids of chloroplasts and its major components. In addition to the mechanisms operating in higher plant chloroplasts, in algae a mechanism for targeting to the pyrenoid is required. The RuBisCO large subunit (LSU) has been shown to be targeted by a co-translational mechanism using FISH (Uniacke and Zerges, 2009), but many factors are still unknown.

Understanding the organelle targeting and protein translocation mechanisms in microalgae is an important tool to enable complex targeting and engineering of recombinant proteins in an algal host cell. Chapter 4 gives an overview how this could be utilised for future biotechnological applications.

1.6. Transition of microalgal culture from laboratory to pilot (and industrial) scale

1.6.1. Upscaling challenges

Translating a microalgal growth system from lab scale to pilot or even industrial scale comes with several challenges that have to be considered carefully and that will vary drastically depending on the species and the system for growth used. The main challenges can be separated into:

a) Engineering of the cultivation unit
b) Physicochemical parameters
c) Biological aspects

On the engineering side, it is crucial to understand that the system has to be feasible and follow the basic laws of thermodynamics. When designing reactors (see section 1.6.2.1), specifically for microalgae, a major constraint for upscaling of systems is light penetration and distribution in a culture vessel. Many factors relating to culture maintenance, control parameters for cultivation units and downstream processing need to be considered when upscaling and are normally neglected in small-scale studies. Physicochemical parameters include the distribution of light, pH control, temperature, aeration and degassing of the cultivation unit. Major challenges are also several biological aspects of the upscaling process – the choice of strain for upscaling will determine what cultivation unit is suitable to grow that specific strain (for example, fragile algae with flagella cannot be cultivated in the same systems as algae with thick cell walls resistant to shear stress). Factors such as inoculum size, formulation of cultivation medium, contamination management and biofilm formation control need to be considered carefully when designing an economically viable and feasible unit.

1.6.2. State of the art in industrial microalgae cultivation

1.6.2.1. Cultivation Systems

Cultivation systems for microalgae are separated into two major categories: open and closed systems. Whereas open systems such as floating devices in the ocean or lakes and ponds (including raceways and cascade systems) are most likely to be used mainly for agricultural purposes in the future, closed systems are of importance for various industrial applications (Verdelho Vieira, 2014). Closed systems include both, fermentation units for heterotrophic growth of algae and photobioreactors (PBR) of various designs, shapes and scale. The most popular design of a PBR is a horizontal tubular version, other designs include flat panel PBRs, airlift PBRs, vertical tubular PBRs, bubble column PBRs and stirred tank PBRs (Gupta *et al.*, 2015). Advantages and limitations of the most common cultivation systems are summarised in Table 1.1.

Table 1.1. Advantages and limitations of the most common cultivation systems for microalgae. Adopted from (Gupta *et al.*, 2015).

System	Mixing	Temp. control	Gas ex-change	Advantages	Limitations
Open pond	Paddle wheel	None	Poor, surface aeration	Cost effective Simple, flexible design Beneficial for mass cultivation	Lower biomass productivity Less control over culturing conditions High contamination risk Need large land space, lower mass transfer Evaporation
Horizon-tal tubular PBR	Recircu-lation via pumps	Shading, overlap-ping, water spraying	Injection into feed, degassing unit	High surface to volume ratio Low hydrodynamic stress Suitable for outdoor cultivation Good biomass productivity Cost effective	Build up of dissolved oxygen Susceptible to photo-inhibition Fouling, biofilm formation Requires large space Poor temp. regulation
Vertical tubular PBR	Airlift/ bubble	None	Open gas exchange at head space	High mass transfer No internal structure Lack of moving parts Good mixing with low shear stress Lower photo inhibition	Low surface area for illumination Expensive material Limited scale up (design constraints) Shading effect
Flat panel PBR	Airlift/ bubble	Heat exchange coils	Open gas exchange at head space	High surface:volume ratio Little space needed High photosynthetic efficiency Cheap and economic Low O_2 build up	Short light penetration depth Not scalable Frequent fouling and clean up issues Poor temp. regulation

1.6.2.2. Downstream processing

The downstream processing of microalgae, i.e. the purification and extraction of algal products, can account for up to 60 % of the total production cost (calculated using EPA recovery from *Phaeodactylum tricornutum* as an example) (Molina Grima *et al.*, 2003), some estimations even stated 80 % of the total production costs (Rasala and Mayfield, 2015). The route chosen to process the algal biomass very much depends on the final product. In general, in a first step the biomass has to be harvested. Several methods can be used to concentrate algal biomass such as: centrifugation, filtration, flocculation, gravity sedimentation, flotation, magnetic separation, electrolysis and ultrasound (Kim *et al.*, 2013). Typically a two-step process is used: first the biomass is separated from bulk suspension and secondly, the slurry is thickened (Brennan and Owende, 2010). After harvesting two major routes are used for further processing: the so-called dry and wet route. Again, the route chosen depends on the biomass characteristics and the end product. Via the dry route, the biomass is dried using e.g. spray drying or freeze drying. If the product needs to be extracted from algal biomass, a step of cell disruption can be necessary. The following methods are used for wet route processing: mechanical disruption (e.g. ultrasound or bead beating), chemical (e.g. organic solvents), biological (using enzymes) or by osmotic shock (Gangl *et al.*, 2015a).

1.6.3. Regulatory aspects of genetically modified algae

To date, there are no algal products on the market made in genetically modified (GM) microalgae. This is at least partly due to the current regulations not being clear on the cultivation of GM microalgae. The main risk associated with GM algae are potential adverse environmental consequences and harm to human and animal health (for food, feed and pharmaceutical applications) (Enzing *et al.*, 2014). An overview of risk assessment for accidental release of GM algae from an enclosed system is shown in Fig. 1.8.

The regulation of GM microalgae can be very different depending on the country – probably with the biggest differences existing between Europe and the USA (Hlavova *et al.*, 2015). In the US the nature of the product rather than the way it is

produced is decisive for GM/non-GM classification: thus the crucial criterion lies on the final product being classified as safe. In contrast in Europe, products made in GM organisms have to be classified as GM food/ GM feed (EC 1829/2003). The cultivation of GM algae and cyanobacteria has to be in a closed system because open pond cultivation would be considered deliberate release (Directive 2009/41/EC, Article 2c) and the setting has to follow the safety level MI-I based on the concept of "Good Industrial Large Scale Practice" (Enzing *et al.*, 2014).

It has been highlighted that avoiding the release of GM algae even in a contained unit will be almost impossible, giving rise to the need for sophisticated risk assessments (Gressel *et al.*, 2013). It is also stressed that most GM algae are expected to have little chance of survival when released (Wijffels *et al.*, 2013) even though others are more concerned about the release of GM algae based on *in silico* analysis and have asked for international regulations (Flynn *et al.*, 2012).

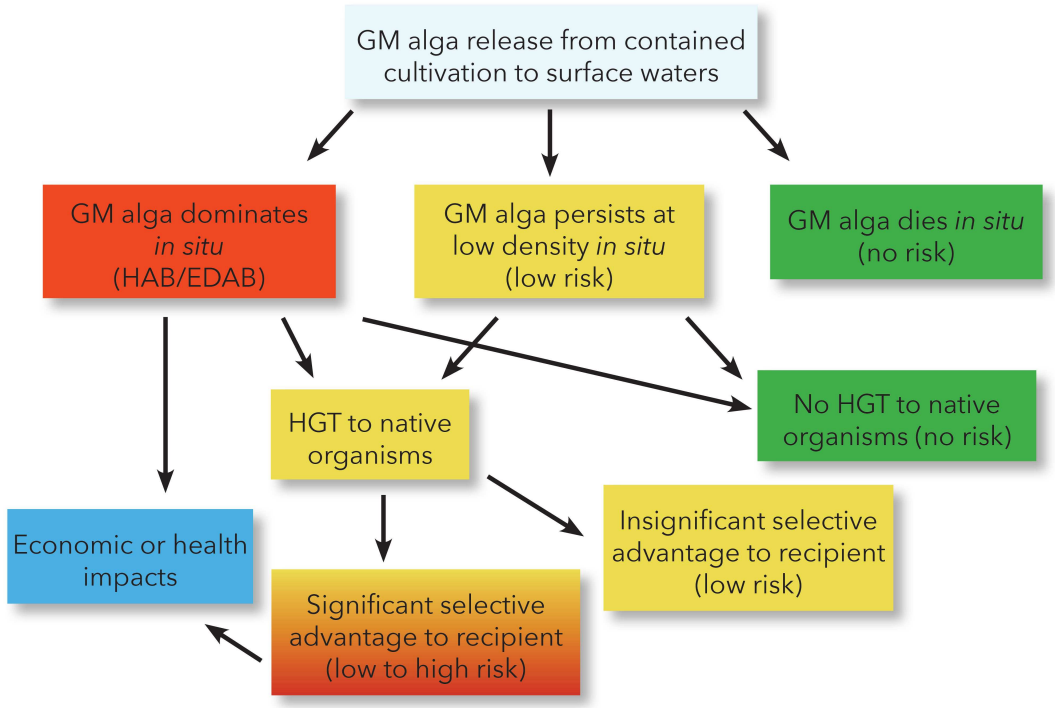


Fig. 1.8. Risk assessment of potential release of GM algae from a cultivation unit. HAB: harmful algal bloom; EDAB: ecosystem disruptive algal bloom; HGT: horizontal gene transfer. Figure adopted from (Henley *et al.*, 2013).

1.6.4. The current market for microalgal products

In 2012, the microalgae business was estimated at 1.325 Million USD per year targeting the aquaculture, food and feed and ceuticals markets (Verdelho Vieira, 2014). The market of microalgae is regarded to be still in its infancy but with a high growth observed since 1999 (Enzing *et al.*, 2014). The worldwide production was estimated at 9000 tonnes per year (Acién Fernández *et al.*, 2013) and biomass costs are still high at 30 to 300 EUR per kg (Brennan and Owende, 2010). The food and feed market is dominated by the production of *Spirulina* sp. and *Chlorella* sp., whereas high-value compounds such as PUFAs (DHA) and pigments (β-carotene, astaxanthin) also contribute majorly to the total market with considerable growth potential. Future developments of microalgae production are thought to be increasingly market driven with a rising need of algal production with a biorefinery concept in order to produce value added products which enable competitive pricing, quality and performance (Verdelho Vieira, 2014).

Over the next years further development can be expected and it remains to be seen if further market sectors, such as pharmaceuticals, can be entered by microalgal products and market segments like novel foods can be expanded. Many challenges including the feasible upscaling of a production system, reduction of biomass production costs and attraction of investment will need to be tackled and will decide about the future role of microalgae on the global market.

1.7. Aims of this thesis

In this thesis an attempt at exploring and expanding the potential of microalgae, more specifically the green alga *Chlamydomonas reinhardtii,* for biotechnological applications is made. Microalgae have been in the spotlight of development as a new biotechnological host for several years now. However, competing with already established platforms such as yeast or mammalian cell culture is still not possible. The idea of having a "green", sustainable platform, a cell factory, which can essentially be driven by light seems attractive but a lot of challenges will have to be overcome to make this economically feasible.

This thesis focuses on exploring unique properties of microalgae that are not found in existing biotechnological hosts. The study in chapter 2 looks at producing a terpene from the secondary metabolism of the conifer *Abies balsamea* in microalgae. Here, the idea is to scavenge the naturally abundant precursor molecule geranylgeranyl diphosphate, from isoprenoid synthesis, for producing high-value compounds using in the chloroplast recombinantly expressed enzymes. The specific terpene, *cis*-abienol is of significance for the perfume industry. In chapter 3 a novel approach of growing transgenic, wall-deficient *Chlamydomonas* strains at pilot scale is introduced. To date, there is only one publication on growth of *C. reinhardtii* at scale, although most studies of microalgae for biotechnological applications are conducted in this organism at lab scale. In this study we wanted to test if it is, at all, possible to grow a wall-deficient, transgenic *Chlamydomonas* strain at scale since there are obvious problems and hurdles. This is one of the first investigations trying to close the gap between lab scale studies and upscaling.

In chapter 4 a closer look at utilising the thylakoid lumen for biotechnological purposes is taken. The thylakoid lumen is a unique compartment that, surprisingly, little is known about. It can offer conditions that are very different from classical biotechnological production platforms and using it effectively could significantly enrich the algal toolkit. Delicate proteins and compounds that are difficult to make in other environments and hosts could be a possible target for this new tool.

Chapter 5 looks at the potential of light-driven product formation in the chloroplast. For this purpose, a model P450, CYP79A1, was chosen for its relocation to the thylakoid membranes in the *C. reinhardtii* chloroplast. The formation of the

product, in this case an oxime, is thought to be light-driven as it scavenges electrons from the photosynthetic electron transport chain, via ferredoxin, for product formation.

All the experiments presented in this thesis have one common goal. The idea of using microalgae as a production host as such is not new, but so far the system is not able to compete with existing platforms and there will still be a long way to go. Rather than developing a "green yeast" the aim of this thesis is to emphasise some of the unique properties of microalgae over other hosts and target those for further investigations. The idea is to develop a biotechnological host that does not attempt to replace other hosts or reproduce what is already optimised in other systems, but rather fill a niche for production of proteins and compounds that can be integrated with the microalgal metabolism where using microalgae has a clear advantage over choosing other hosts.

Chapter 2: Stable expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas reinhardtii*

Zedler, J.A.Z., Gangl, D., Hamberger, B., Purton, S., and Robinson, C. (2015) Stable expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas reinhardtii*. *J Appl Phycol* **27:** 2271–2277.

2.1. Author contribution

I am the first author of this research article. I designed the experiment with the help of CR, SP and BH. I undertook all the experimental work and collected the data (cloning of the constructs, generation of chloroplast transformants, analysis of chloroplast transformants, purification of the TPS4 protein, growth studies). Trypsin in-gel digestion of the purified TPS4 and MS analysis was done by Kevin Howland (University of Kent). All results and data were interpreted by me and discussed with the other authors of the research article. I wrote the manuscript together with CR and generated all figures apart from Supplementary Figure 2 (compiled by BH). The manuscript was edited and approved by all other authors of the research study.

2.2. Aim of this study

This study was designed as a proof of concept. To date, there were no studies about the synthesis of a diterpene in microalgae. Typically, the synthesis of a diterpene in plants is a complex metabolic pathway that requires several catalytic steps and involves multiple enzymes which represents a major challenge for genetic engineering of those pathways into a desired host (Pateraki *et al.*, 2015). In this regard, the enzyme chosen for this study is rather unique. *Cis*-abienol synthase (TPS4) from *Abies balsamea* has previously been expressed recombinantly in *E. coli.* Due to its bifunctional catalytic activity only this single enzyme is required to catalyse the formation of *cis*-abienol from GGPP (Pateraki *et al.*, 2015). GGPP is a precursor molecule abundant in the chloroplast of *C. reinhardtii* (see Section 1.5.1 for details). Therefore recombinant expression of TPS4 should be sufficient for *in vivo cis*-abienol synthesis.

This proof of concept study aimed at setting the scene for terpene production in microalgae. Choosing a simple pathway from *Abies balsamea* allowed circumventing complex pathway engineering in the chloroplast and delivered at the same time a high-value molecule that is of true biotechnological interest.

2.3. Original research publication

Remark: Here, the pre-proof version of the manuscript is presented. The references have been added to the bibliography in chapter 7, the figures and tables numbering and position as well as headings have been changed for consistent integration into this thesis, but the content was not modified.

2.3.1. Abstract

Chlamydomonas reinhardtii has been shown to hold significant promise as a production platform for recombinant proteins, but transformation of the nuclear genome is still a non-trivial process due to random gene insertion and frequent silencing. Insertion of transgenes into the chloroplasts is an alternative strategy, and we report here the stable expression of a large (91 kDa) protein in the chloroplast using a recently developed low-cost transformation protocol. Moreover, selection of transformants is based on restoration of prototrophy using an endogenous gene (*psbH*) as the marker, thereby allowing the generation of transgenic lines without the use of antibiotic-resistance genes. Here, we have expressed a bifunctional diterpene synthase in *C. reinhardtii* chloroplasts. Homoplasmic transformants were obtained with the expressed enzyme accounting for 3.7 % of total soluble protein. The enzyme was purified to homogeneity and expression was shown to have a small but reproducible effect on growth rate at the end of log phase growth. These results demonstrate that large recombinant enzymes can be synthesised in the algal chloroplast, and serve to underline its potential as a platform for the biosynthesis of novel metabolites.

Keywords

Chlamydomonas, Chlorophyta, chloroplast transformation, recombinant protein, diterpene synthase, glass bead, endogenous marker

2.3.2. Introduction

A number of reports have demonstrated the potential of microalgae as biotechnological platforms for recombinant protein production. *Chlamydomonas reinhardtii* has been a popular host strain, because genetic tools are relatively advanced, and recombinant protein targets have included vaccines, antibodies, hormones and immunotoxins (Gregory *et al.*, 2012; Demurtas *et al.*, 2013; Gregory *et al.*, 2013; Jones *et al.*, 2013; Tran *et al.*, 2013a; Tran *et al.*, 2013b; Soria-Guerra *et al.*, 2014). However, attempts have also been made to further develop the platform for other applications, such as industrial enzymes (Rasala *et al.*, 2012; Pourmir *et al.*, 2013) and functional food supplements (Campos-Quevedo *et al.*, 2013) or the production of biodegradable plastics (Chaogang *et al.*, 2010) (reviewed in (Rosales-Mendoza *et al.*, 2012; Purton *et al.*, 2013; Specht and Mayfield, 2014)).

Many such studies have relied on transformation of the nuclear genome for recombinant protein expression, but DNA insertion into the genome is essentially random. Therefore, levels of transgene expression are variable and unpredictable owing to position effects, and frequently unstable because of gene silencing (Rosales-Mendoza *et al.*, 2012). Furthermore, for metabolic engineering strategies where recombinant enzymes are required to localise to the chloroplast, nuclear transformation requires that cytoplasmically synthesised proteins are targeted into the chloroplast by means of a 'transit peptide'. Insertion of transgenes into the chloroplast genome is thus an attractive alternative approach, particularly since genes can be targeted to specific loci via homologous recombination and high-level and stable expression can be achieved without any evidence for gene silencing (reviewed in (Purton *et al.,* 2013, Specht and Mayfield, 2014)). Genes are typically introduced by particle bombardment and, although chloroplast transformation by agitation with glass beads was reported some time ago (Kindle *et al.*, 1991), very few studies have used this method (Demurtas *et al.*, 2013, Economou *et al.*, 2014).

Recently, a novel method of chloroplast transformation was reported, which combines a rapid and low-cost glass bead-based transformation approach with a novel selection method. In this approach, insertion of the transgene into the chloroplast genome is accompanied by restoration of an intact chloroplast *psbH* gene, and selection is based on restored photoautotrophic growth. The procedure uses a cell-wall mutant

recipient strain in which the chloroplast *psbH* gene is disrupted by an *aadA* antibiotic cassette and is incapable of photoautotrophic growth (Economou *et al.*, 2014). The pASapI transformation vector bears a functional copy of *psbH* with the gene of interest inserted into an intergenic region immediately downstream, and integration into the chloroplast genome via homologous recombination simultaneously introduces both *psbH* and the gene of interest. Insertion of the transgene into the chloroplast genome is accompanied by restoration of an intact chloroplast *psbH* gene that encodes an essential subunit of photosystem II (O'Connor *et al.*, 1998) with selection based on restored photoautotrophic growth. Importantly, the *aadA* cassette is removed during this process and is completely absent in transformants that have homoplasmic chloroplast DNA copies. This method thus represents a relatively simple transformation system, in which any gene of interest can be precisely inserted and expressed in a comparably short timeframe.

In this study, we report the expression of a synthetic gene encoding a bifunctional diterpene synthase of 91 kDa. Diterpene synthases are key enzymes in the synthesis of a wide range of terpenes, many of which have been used for medical and other purposes, and in this study, we expressed *cis*-abienol synthase from Balsam fir. The enzyme catalyses the formation of *cis*-abienol from the abundant precursor molecule geranylgeranyl diphosphate (Zerbe *et al.*, 2012) and *cis*-abienol has potential as a source for the synthesis of Ambrox, an important compound in the perfume industry. We show that the enzyme accumulates in the chloroplast stroma of transformed *Chlamydomonas* strains, accounting for approximately 3.7 % of total soluble protein, and expression has a minor but detectable effect on growth rate at the end of log phase growth. To our knowledge, this is the largest single protein produced to date in the *C. reinhardtii* chloroplast, and indicates that novel metabolic pathways using multi-functional enzymes could be engineered into the organelle.

2.3.3. Materials and methods

2.3.3.1. Plasmid construction

For plasmid construction, the vector pASapI was used (Economou *et al.*, 2014). A synthetic TPS4 gene from *Abies balsamea* was designed using the primary sequence

reported for recombinant expression in *Escherichia coli* in which the mature sequence, lacking the plant chloroplast transit sequence, is predicted to start at residue 87 (GenBank accession No. AEL99953.1; Zerbe *et al.*, 2012). An HA tag was added at the C-terminus to allow detection of the protein. The gene sequence was codon-optimised for expression in the *Chlamydomonas* chloroplast using the software 'Codon Usage Optimizer' (codonusageoptimizer.org/download) and synthesised de novo by GenScript (USA). The TPS4-HA sequence was cloned into the pASapI vector at the *SapI* and *SphI* sites. The plasmid construct, termed pJZ2a, was confirmed by sequencing using the primers atpA.F and rbcL.R (Economou *et al.*, 2014) amplified in *E. coli* DH5α cells and used for *Chlamydomonas* chloroplast transformation.

2.3.3.2. Cultivation of *Chlamydomonas* strains and transformation

The *C. reinhardtii* strain CC-125 (mt+), used as a wild-type control, was kindly provided by Alison Smith (University of Cambridge, UK). The *C. reinhardtii* strain TN72 (cw15, *psbH::aadA*, mt+) used for transformation was created in the Purton lab. All strains were cultivated at 25 °C with approx. 50 µmol photons m^{-2} s^{-1} continuous illumination on agar plates. Liquid cultures were incubated in a Multitron Pro Shaking Incubator (Infors Ltd.) at 110 rpm, 25 °C and approximately 15 µmol photons m^{-2} s^{-1}. Cultures were grown mixotrophically in tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) with a modified trace metal recipe (Kropat *et al.*, 2011).

A glass bead transformation method as described by Economou *et al.* (Economou *et al.*, 2014) was used for transformation. Mid-log phase cells of the strain TN72 were transformed with the constructs pJZ2a or the pASapI vector lacking a gene insert. For selection of transformants, the minimal medium high salt medium (HSM) (Sueoka, 1960) with a modified trace metal recipe (Kropat *et al.*, 2011) was used. After re-streaking the transformant to single colonies six times on HSM, homoplasmy of the cell lines was confirmed by PCR. An overview of the transformation procedure is given in Online Resource 1.

2.3.3.3. DNA extraction and PCR analysis of transformants

Total genomic DNA of single colonies was extracted using a Chelex 100 Resin (Bio-Rad) using the protocol described in Economou *et al.* (2014). Homoplasmy was confirmed by PCR with three different primers using the principle described by Economou *et al.* (2014). For pASapI transformants, the primers used were:

(i) FLANK1 (GTCATTGCGAAAATACTGGTGC). Anneals downstream of the gene of interest (GOI) insertion site, just beyond the 0.8-kb region of homology carried on the pASapI vector

(ii) rbcL.F (CGGATGTAACTCAATCGGTAG). Anneals to the end of the *aadA* cassette inserted within the TN72 genome and gives a 0.85-kb product in conjunction with FLANK1 for the untransformed TN72 genome

(iii) atpA.R (ACGTCCACAGGCGTCGTAAGC). Anneals to the *atpA* promoter/5′UTR element-driving expression of the GOI and gives a 1.20-kb product in conjunction with FLANK1 for the transplastomic genome

A second PCR reaction was run to screen for the loss of the *aadA* cassette upon transformation using the primers atpA.F and rbcL.R (Economou *et al.*, 2014). For all PCR reactions, a standard PCR protocol with Phusion Hot Start Flex Polymerase (New England Biolabs) was used. For the three primer PCRs, an annealing temperature of 65.2 °C was used. In the *aadA* cassette PCR with the primers atpA.F and rbcL.R, the annealing temperature was 62 °C.

2.3.3.4. Protein expression in *E. coli* and *Chlamydomonas*, SDS-PAGE and Western blot analysis

For the preparation of *Chlamydomonas* lysates, cells were lysed by sonication in lysis buffer (20 mM HEPES pH 7.2, 5 % glycerol, 20 μg mL^{-1} DNAse (Roche), 1/2 tablet EDTA-free protease inhibitor tablet (Roche) per 20 mL). Five equivalents of milligram chlorophyll per litre culture were separated on a 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-HA-antibody (Sigma-Aldrich). The signal was detected with an ECL reagent

(Biological Industries) and imaged using a ChemiDoc XRS+ System (Bio-Rad). For expression studies in *E. coli*, pASapI and pJZ2a were transformed into *E. coli* MC4100. The TPS4-HA protein was expressed overnight at 16 °C in TB medium based on a protocol described elsewhere (Keeling *et al.*, 2008). For cell lysis, the *Chlamydomonas* buffer was used, additionally containing 0.1 mg mL^{-1} lysozyme (Sigma-Aldrich). The further procedure was identical as for the algal samples.

2.3.3.5. TPS4-HA purification

The TPS4-HA protein was purified from 5 L of late log phase *Chlamydomonas* TN72-TPS4 culture. Cells were harvested at 4000 rpm, 4 °C for 20 min in a Beckman Coulter Avanti J-26SXP centrifuge and pellets were resuspended in lysis buffer (see above). Lysates were sonicated and cleared by ultracentrifugation at 70,000 rpm, 4 °C and 30 min (Beckman TL-100 ultracentrifuge). The soluble supernatant was loaded onto five 5-mL Q-Sepharose columns (Q-Sepharose Fast Flow, GE Healthcare), which were washed in 20 mM HEPES pH 7.2, 5 % glycerol, 1 mM $MgCl_2$ and 5 mM dithiothreitol (DTT). The TPS4-HA protein was eluted using two column volumes of the same buffer containing 350 mM NaCl, which was specifically optimised for this protein. The elution fractions were concentrated using Vivaspin 20 columns (GE Healthcare) and subjected to affinity chromatography using 2 mL Pierce Anti-HA Agarose resin (Thermo Scientific) using the manufacturer's protocol. The TPS4-HA protein was eluted with an HA competitor peptide (Thermo Scientific), and purity was confirmed by SDS-PAGE and subsequent Oriole fluorescent gel (Bio-Rad) staining and immunoblotting.

2.3.3.6. Peptide mass fingerprinting: peptide preparation and analysis

The purified TPS4-HA protein was run on a SDS-PAGE. After Coomassie staining, the TPS4-HA band was excised and dissected into approximately 1 mm-sized pieces. After reduction of disulphide bonds with DTT and alkylation of cysteine residues with chloroacetamide, the sample was digested overnight with trypsin.

Generated peptides were extracted and analysed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The generated mass fingerprint was searched against the SWISS-PROT Database using the MASCOT search engine. The 15 most intense peptide masses were then subjected to further analysis by MS/MS and the obtained data searched against the same database.

2.3.3.7. Protein quantitation

Ten millilitres of late log phase cultures of the TN72-TPS4 strain were harvested and lysed. The total soluble protein content of these samples was determined using the *DC* Protein Assay Kit (Bio-Rad) following the manufacturer's instructions. Bovine serum albumin (Bio-Rad) was used as a standard. The same lysate was subjected to SDS-PAGE and Western blot analysis. A recombinant human HA-tagged Ubiquitin protein (Boston Biochem) was loaded in known quantities as a calibration standard. Densitometric analysis was performed using ImageLab 4.1 software (Bio-Rad).

2.3.4. Results

2.3.4.1. Transformation strategy

A synthetic gene encoding the mature sequence of the bifunctional *cis*-abienol synthase TPS4 from the balsam fir tree, *A. balsamea*, was designed based on optimal codon usage in the *C. reinhardtii* chloroplast, and with the 3′ end of the coding sequence extended to encode the hemagglutinin epitope. The gene was inserted into the chloroplast expression vector pASapI, generating construct pJZ2a as shown in Fig. 2.1, with the *TPS4* gene driven by the chloroplast *atpA* promoter and 5′ untranslated region. A glass bead-based vortexing method was then used to transform the chloroplast genome of the photosynthetic mutant TN72 lacking an intact *psbH* gene as detailed in 'Materials and Methods' and illustrated in Online Resource 1. Following selection for photoautotrophic growth on minimal medium, a transformed line with restored photo-autotrophic growth was obtained (termed TN72-TPS4). An additional strain with

restored photoautotrophy, TN72-RP, was created for control purposes by transformation using the pASapI vector lacking any insert.

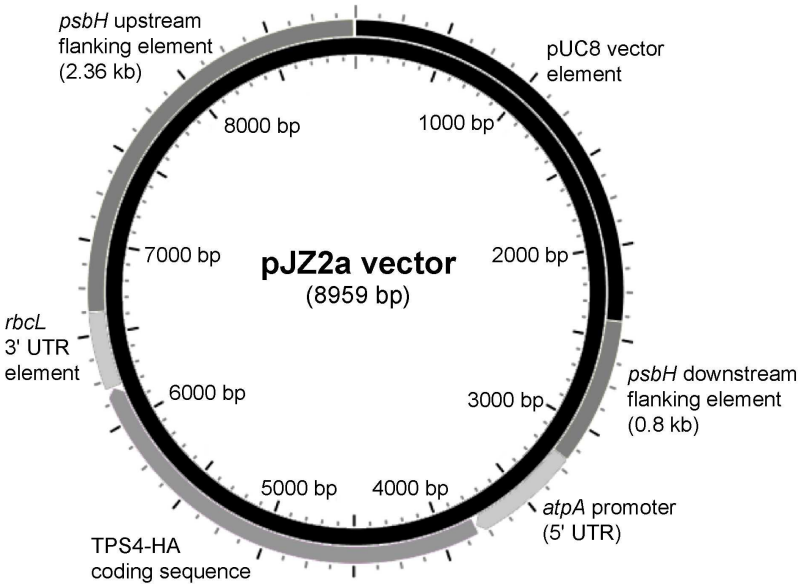


Fig. 2.1. Schematic map of the plasmid pJZ2a. The pJZ2a vector is based on the plasmid pASapI (Economou *et al.*, 2014). A codon-optimised sequence of the abienol synthase gene *tps4* was cloned into the vector using the *SapI* and *SphI* sites. An HA tag coding sequence was appended at the 3′ end of the *tps4* gene. Successful transformation of *Chlamydomonas* is achieved upon homologous recombination of the *psbH* flanking regions. The heterologous gene is driven by the endogenous *atpA* promoter. The *psbH* upstream-flanking element has a functional copy of *psbH*. The pUC8 vector fragment allows replication of the plasmid in *E. coli*. This plasmid map was assembled using PlasMapper Version 2.0 (modified).

Insertion of the gene into the chloroplast genome, and the resulting loss of the *aadA* cassette, was confirmed by PCR analysis as shown in Fig. 2.2. Panel A shows PCR reactions carried out using primers that amplify a 1.1-kb region of the '*aadA* cassette' present in the genome of the TN72 recipient strain (Economou *et al.*, 2014). This band is present in the TN72 sample but absent in the TN72-TPS4 transformant and TN72-RP restored wild type, suggesting that the cassette has been lost as a result of transformation. This is further confirmed in panel B where a combination of three PCR primers was used to test each strain for the presence of either type of genome. A primer to the downstream flanking region in combination with a primer to the *aadA* cassette in TN72 gives a 0.85-kb product, whereas the flanking primer together with one to the

atpA promoter/5′UTR element linked to the gene of interest gives a 1.2-kb product. The results show that the 1.2-kb band is seen for the TN72-RP and TN72-TPS4 transformants, and the 0.85-kb band is seen for the host TN72 strain. Importantly, the absence of the 0.85-kb band in the transformants confirms that the chloroplast DNA copies are homoplasmic, such that all of the *psbH::aadA* copies have been replaced with a functional *psbH* together with the transgene cassette. No heteroplasmic cell lines were generated.

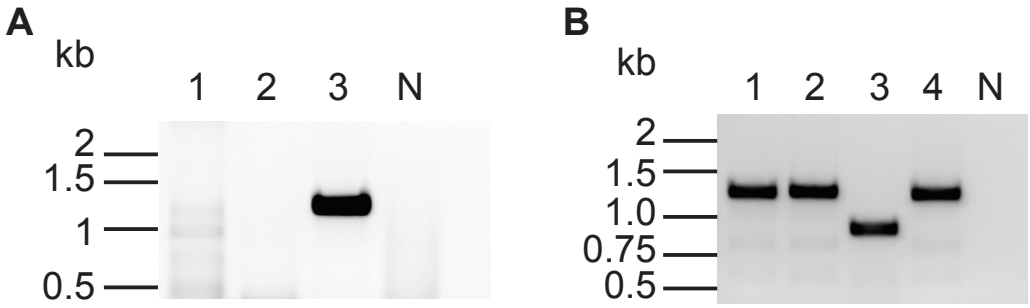


Fig. 2.2. Transformation results in homoplasmic insertion of the *tps4* gene. Total DNA was extracted from TN72-TPS4 and TN72-RP cells and subjected to PCR analysis using primers that amplify a 1.1 kb region including the *aadA* cassette in the host strain (3), which was lost upon homologous recombination in the transformants TN72-TPS4 and TN72- RP (1, 2) (**A**). **B** shows the amplification of a 1.2-kb region including the restored *psbH* gene in transformed copies of the chloroplast genome, whereas a 0.85-kb fragment is amplified in untransformed copies of the chloroplast genome. The figure shows PCR data for the TN72-TPS4 and TN72-RP transformants (1, 2), the TN72 host strain (3), a positive control (4) and a control reaction using H_2O in place of DNA (N). Mobilities of the 0.75, 1.0 and 1.5 kb marker bands are indicated.

2.3.4.2. Expression of the TPS4 protein

TPS4 expression in the transformant TN72-TPS4 was detected by immunoblot analysis using an anti-HA antibody (Fig. 2.3). The ‘TPS4’ lane shows the presence of an approximately 80-kDa band, which is smaller than the predicted size of the TPS4-HA protein (around 91 kDa). A protein of the same size is also detected in the *E. coli* strain containing the pJZ2a vector (‘*E. coli*’ lane), strongly suggesting that the protein runs aberrantly on SDS gels. The protein is absent in samples from CC-125 wild-type

cells (WT) or the control transformant generating restored photosynthetic competence (RP). The antibody does react with an endogenous protein of about 30 kDa in all *Chlamydomonas* samples tested, showing that this is a non-specific reaction.

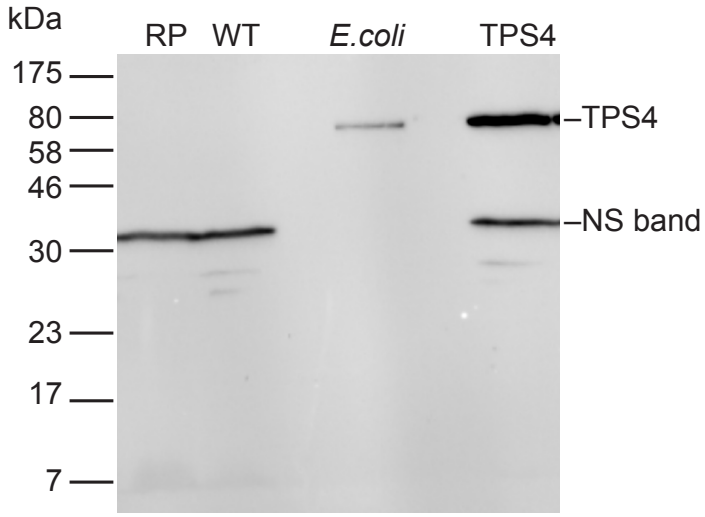


Fig. 2.3. Abienol synthase (*tps4*) is expressed in *Chlamydomonas reinhardtii* TN72-TPS4 cells. Extracts of *Chlamydomonas* cells, and of the *E. coli* strain expressing pJZ2a, were subjected to immunoblotting using antibodies to the C-terminal HA-tag. The blot shows data for TN72-RP strain (RP), the wild-type strain CC-125 (WT), *E. coli* host expressing pJZ2a (*E. coli*) and TN72-TPS4 transformant (TPS4). The TPS4-HA protein is indicated; NS non-specific reacting band. Mobilities of molecular mass markers (in kDa) are indicated on the left.

To further confirm that the approximately 80-kDa protein does bear an HA tag, and is therefore TPS4-HA, we purified the protein as shown in Fig. 2.4. Soluble cell extracts were first subjected to ion exchange chromatography (Fig. 2.4a) and the panel shows an immunoblot of initial lysate (Lys), wash fractions (W) and the fraction eluted with 350 mM NaCl (E). The data show that the TPS4-HA protein is present in the elution fraction while the non-specifically reacting 30-kDa protein is present in the flow-through and wash fractions. The 350 mM NaCl eluate was then applied to an HA-affinity column (Fig. 2.4b), and the panel shows an Oriole-stained gel of the flow-through and wash samples, with the purified TPS4-HA present in the elution fractions. These data show that this protein can be expressed in *Chlamydomonas* chloroplasts and purified to homogeneity.

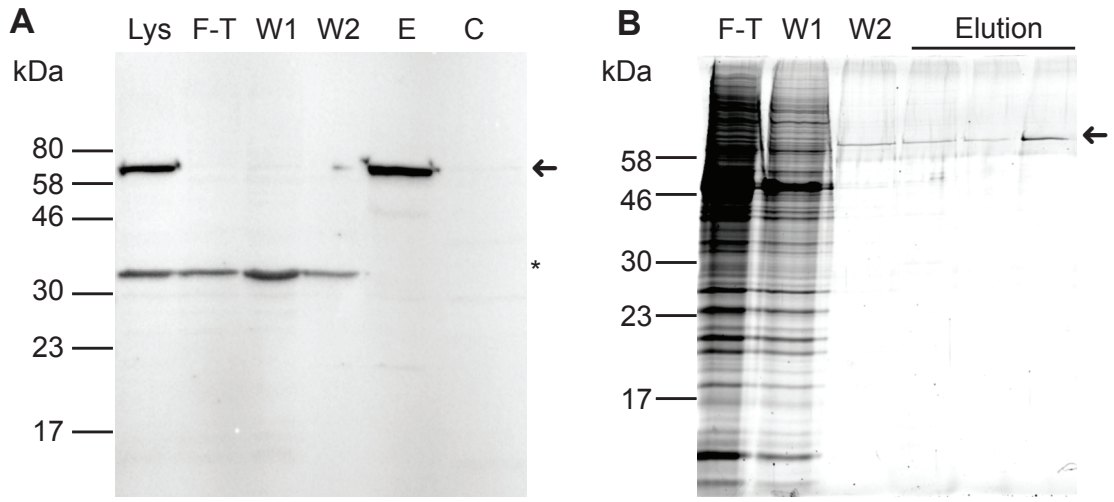


Fig. 2.4. Purification of TPS4-HA from total soluble protein. **A** Immunoblot analysis of ion exchange chromatography elution fractions; the panel shows results for the total cell lysate (Lys), column flow-through (F-T), wash fractions (W1, W2) and the 350-mM NaCl elution fraction (E) containing TPS4-HA. Mobilities of molecular mass markers (in kDa) are indicated. **B** Oriole^{TM}-stained SDS gel of TPS4-HA affinity purification fractions showing column flow-through (F-T), wash fractions (W1, W2) and elution fractions obtained using the competitor HA-peptide. Arrows denote TPS4 protein; an asterisk denotes non-specific reacting band in A.

Furthermore, the purified TPS4-HA protein was subjected to peptide mass finger print analysis. The protein CAS_ABIBA was identified unambiguously (Score 191 searched against the SWISS-PROT Database, p=4.3e^{-14}). The heterologous TPS4-HA protein of this study is identical in its amino acid sequence to CAS_ABIBA (*cis*-abienol synthase from Balsam fir), apart from removal of the N-terminal plastid transit sequence for expression in chloroplasts (shown in Online Resource 2, which also shows the homology exhibited between TPS4 and other diterpene synthases) and addition of a C-terminal HA tag. Details of the mass spectrometry analysis are given in Online Resource 3, where coverage of the protein sequence and details of the identified peptides are shown.

2.3.4.3. Quantitation of protein levels

In order to quantitate the levels of TPS4-HA in the transformant, we carried out densitometric analysis of immunoblots using purified HA-Ubiquitin (containing an identical HA tag to that of TPS4-HA) as a standard for the calibration curve. The expressed TPS4 enzyme was found to represent approximately 3.7 % of total soluble protein. In general, similar expression levels (in terms of percentage of total soluble protein) were obtained for both photoautotrophic growth on minimal medium and mixotrophic growth on TAP medium that contains acetate as a source of fixed carbon (data not shown).

2.3.4.4. Expression of TPS4 has a minor adverse effect on growth rate

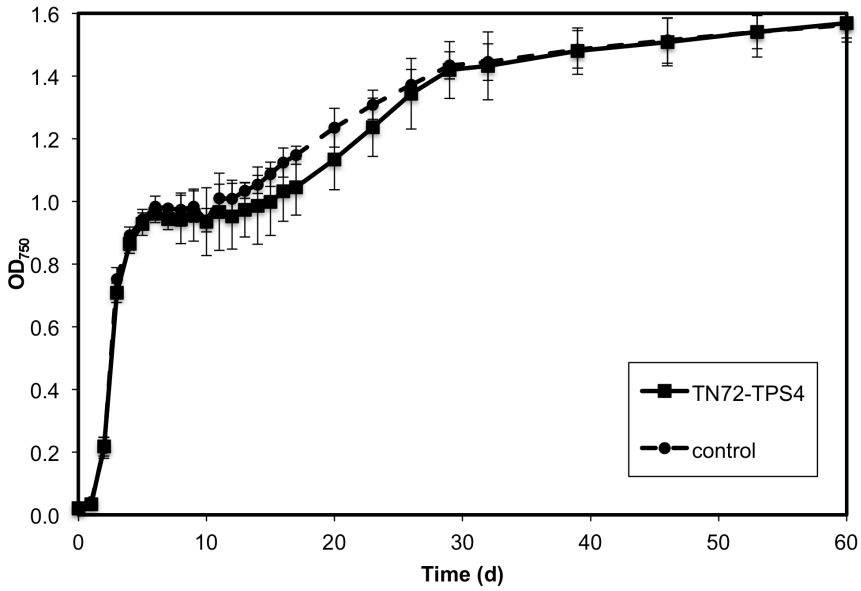


Fig. 2.5. Expression of the *tps4* transgene has a minor effect on culture growth. The graph shows growth curves for TN72-RP (*circles, dashed line*) and TN72-TPS4 cultures (*boxes, continuous line*) on TAP medium. The data are averages of three experiments.

TPS4 catalyses the formation of *cis*-abienol using geranylgeranyl diphosphate (GGDP) as substrate (Zerbe *et al.*, 2012), but we were unable to detect *cis*-abienol in the cultures of the transformant strain so it is unclear whether the enzyme is active in the transformed cells. However, expression of the enzyme may be expected to partially

deplete the GGDP pool in the chloroplast and therefore affect growth of the strain, and this was tested by directly comparing the growth of TN72-TPS4 and the TN72-RP control strain. The cultures were grown on TAP medium, and the growth curves are shown in Fig. 2.5. Both exhibit rapid initial log growth characteristics that appear to be identical; thereafter, the cultures enter a steady linear phase, which presumably occurs after depletion of the acetate. At this point, growth of the TN72-TPS4 culture is slightly slower than that of the TN72-RP culture, suggesting that TPS4 activity may indeed be depleting the GGDP pool and thereby inhibiting GGDP-dependent metabolic pathways in the chloroplast.

2.3.5. Discussion

A recent study has shown that a relatively simple method can be used to transform the chloroplast genome of *C. reinhardtii* (Economou *et al.*, 2014). In the present study, we sought to test the utility of this method for the expression of heterologous proteins, using a plant diterpene synthase as target protein. For this study, a gene encoding the bifunctional enzyme TPS4 was integrated into the chloroplast genome and the cells were characterised once homoplasmy was achieved.

Immunoblotting showed that the 91-kDa HA-tagged TPS4 protein is stably expressed, and it was furthermore shown that the protein can be purified to homogeneity from cell extracts. MALDI-TOF mass fingerprinting further confirmed the identity of the protein sequence. We calculated that the protein represents 3.7 % of total soluble protein. Together, these data show that the transformation method is suitable for expression at reasonably high levels of a heterologous protein and it should be possible to further enhance expression levels by manipulating promoter regions and other UTR elements. Importantly, the method does not result in the presence of an antibiotic cassette in the chloroplast genome of transformed cells and this may be an advantage in terms of minimising the effort needed to generate and maintain the cultures. The absence of such cassettes is also preferred for commercial production applications.

No clear phenotype was observed for the transformant expressing TPS4 and we did not detect the reaction product, *cis*-abienol (Zerbe *et al.*, 2012), using gas chromatography mass spectrometry. However, this product is not available in purified

form for control tests and the compound may well have been masked by other compounds in our tests. We did note that the transformed strain grew slightly more slowly than the mock-transformed control strain, which may be an indirect indication of enzyme activity, but this point remains to be resolved. This enzyme has been studied in *E. coli* but only using *in vitro* assays (Zerbe *et al.*, 2012), and this is the first report of heterologous expression in photosynthetic hosts; it is therefore difficult to predict the effects of product accumulation. Other studies (Jin *et al.*, 2014) have shown that synthesis of a variety of hydrophobic compounds can adversely affect growth of photosynthetic bacteria, and the observed change in growth of this TPS4 transformant may well reflect this trend.

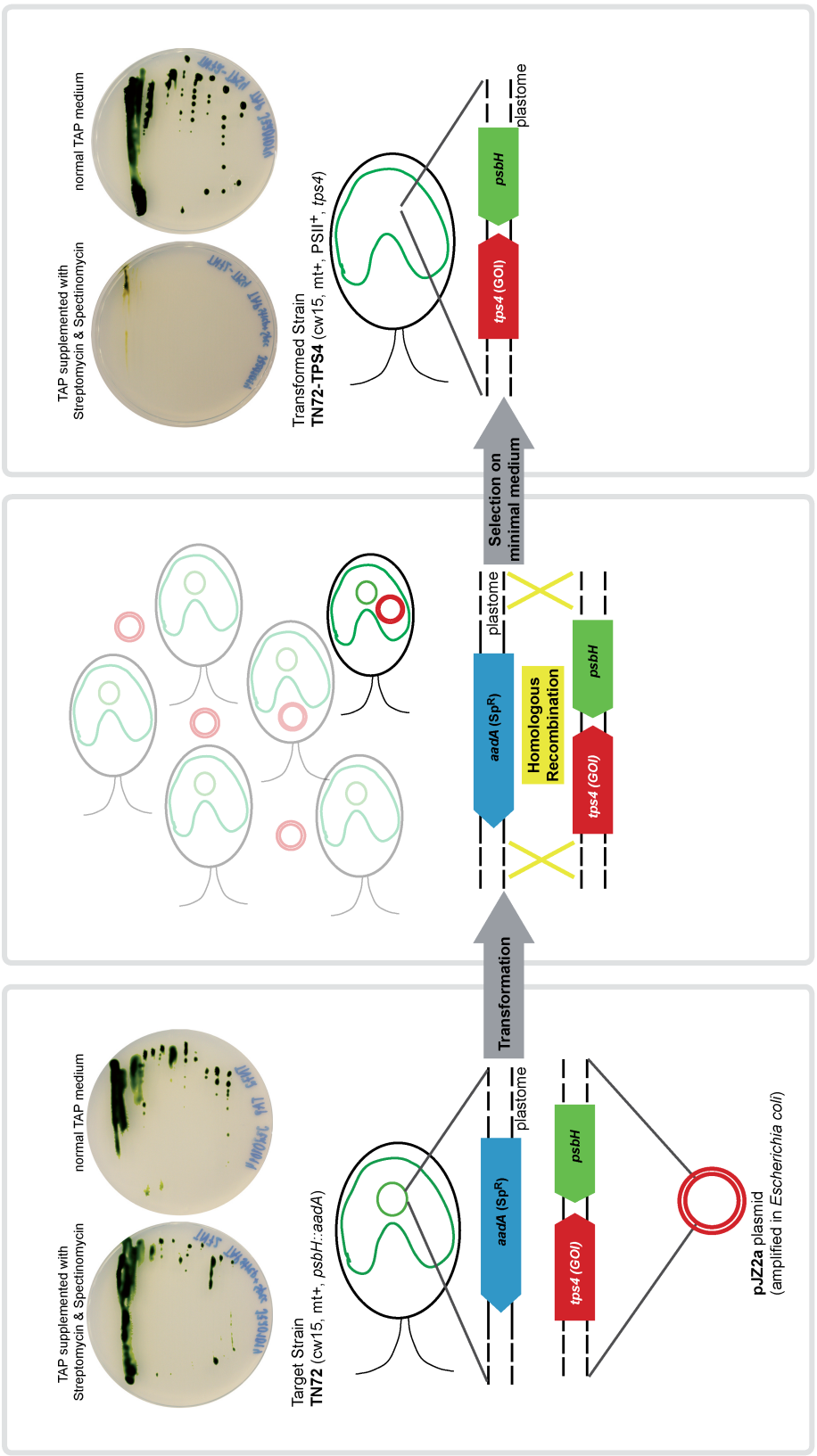
In summary, the result show that this recently developed transformation protocol is capable of generating strains that stably express a heterologous protein in the chloroplast at levels that are generally comparable with other studies using either nuclear or chloroplast transformation.

2.3.6. Acknowledgments

The authors thank Kevin Howland (Biomolecular Science Facility, School of Biosciences, University of Kent) for his help with peptide mass fingerprint analysis and Umaima Al-Hoqani for the codon optimisation of *tps4*. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement no. 317184.

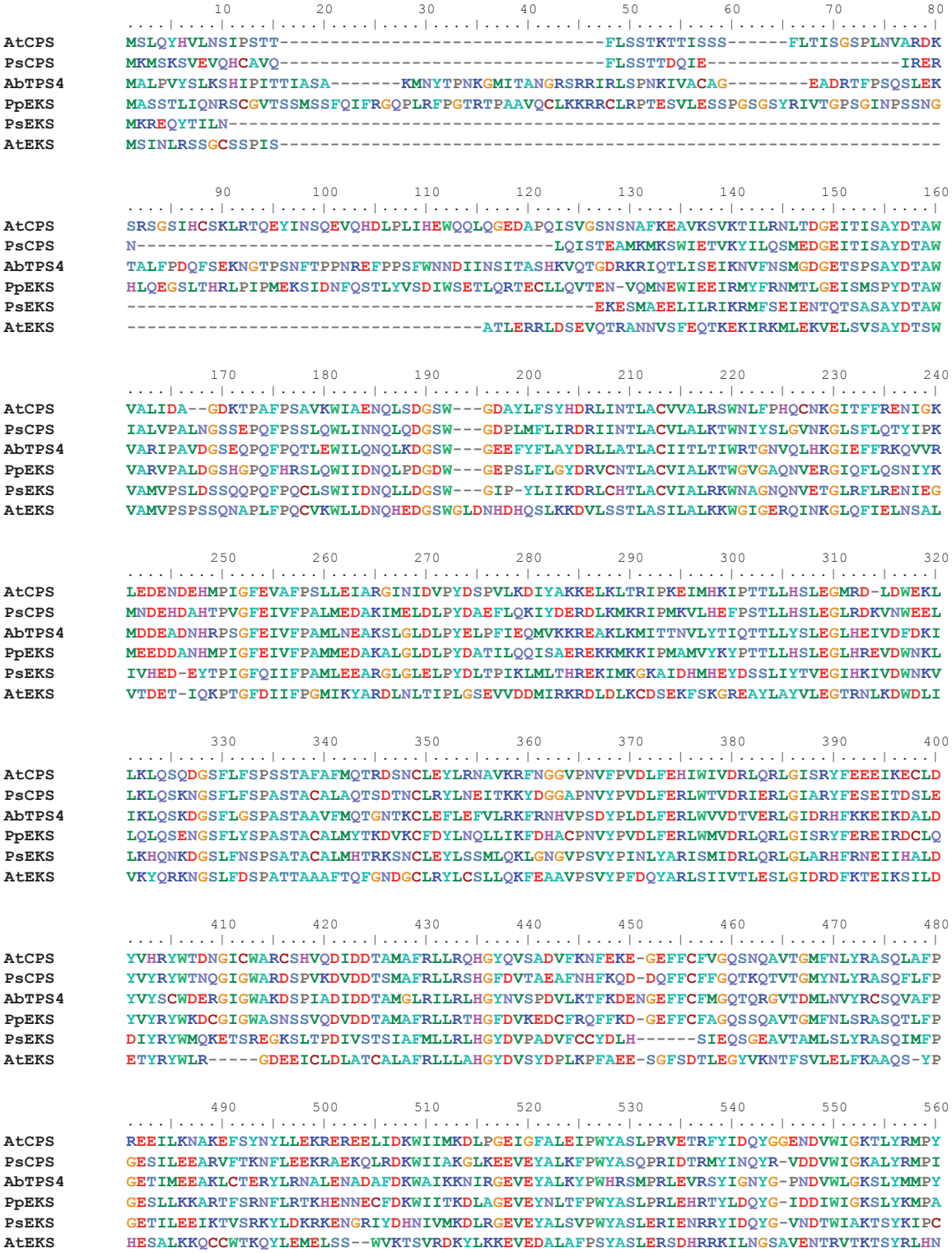
2.3.7. Supplementary material

2.3.7.1. Online resource 1



Online Resource 1. Method for Chloroplast Transformation and Selection of Transformants in *Chlamydomonas reinhardtii* using the pASapI vector. The target strain TN72 for chloroplast transformation has an *aadA* cassette inserted. The *psbH* gene is deleted thus photosystem II is not functional. TN72 strain cells can grow on streptomycin and spectinomycin supplemented medium with a carbon source (as *e.g.* TAP), but not on minimal medium due to their lack of photosynthetic capacity. Upon transformation TN72 cells and pJZ2a vector DNA (pASapI vector with tps4 gene integrated) are vortexed with glassbeads. The vector DNA will then get incorporated in some of the TN72 cells and upon successful homologous recombination with the plastome of the target strain, a functional copy of *psbH* as well as the gene of interest, *tps4*, gets integrated into the plastome. To enforce restoration of photosynthetic capacity, the cells are selected for autotrophic growth on minimal medium. The obtained transformants lost the *aadA* cassette by recombination and therefore can't grow on TAP medium supplemented with streptomycin and spectinomycin. A detailed description of the applied transformation method is given in Economou et al. 2014.

2.3.7.2. Online resource 2



Online Resource 2. Alignment of plant diterpene synthases of general and specialised metabolism.
Shown are the monofunctional angiosperm *Arabidopsis* and gymnosperm *Sitka spruce* (*Picea sitchensis*) copalyl-diphosphate synthases (class II, AtCPS, PsCPS) and ent-kaurene synthases (class I, AtEKS, PsEKS), the bifunctional ancient progenitor, ent-kaurene synthase from the moss *Physcomitrella patens* (class II/I, PpEKS), as well as the gymnosperm balsam fir diterpene synthase (class II/I, AbTPS4). Indicated by the triangle is the predicted plastidial target sequence in AbTPS4, highlighted are the conserved aspartate rich domains characteristic for class II (DxDD) and class I diTPS (DDxxD). Both domains are present in the moss and balsam fir diterpene synthases.

a **CAS_ABIBA**

MS: Score: 191 Expect: $4.3 \cdot 10^{-14}$ Sequence Coverage: 50%
MS/MS: Score: 392 Sequence Coverage: 12%

MALPV YSLKS HIPIT TIASA KMNYT PNKGM ITANG RSRRI RLSPN KIVAC
*AGEAD RTFPS QSLEK TALFP DQFSE KNGTP SNFTP P*NREF PPSFW NNDII
NSITA SHKVQ TGDRK **RIQTL ISEIK NVFNS MGDGE TSPSA YDTAW VAR**IP
AVDGS EQPQF PQTLE WILQN QLK**DG SWGEE FYFLA YDR**LL ATLAC IITLT
IWRTG NVQLH K**GIEF FRK**QV VR**MDD EADNH RPSGF EIVFP AMLNE AKSLG**
LDLPY ELPFI EQMVK KREAK LKMIT TNVLY TIQTT LLYSL EGLHE IVDFD
KIIKL QSK**DG SFLGS PASTA AVFMQ TGNTK CLEFL EFVLR** KFR**NH VPSDY**
PLDLF ERLWV VDTVE RLGID RHFKK EIK**DA LDYVY SCWDE R**GIGW AK**DSP**
IADID DTAMG LRILR LHGYN VSPDV LKTFK DENGE FFCFM GQTQR **GVTDM**
LNVYR CSQVA FPGET IMEEA KLCTE RYLR**N ALENA DAFDK WAIK**K **NIRGE**
VEYAL KYPWH RSMPR LEVR**S YIGNY GPNDV WLGKS LYMMP YISNE KYLEL**
AKLDF NSVQS LHQEE IRELV RWCK**S SGFTE LKFTR** DRVVE TYFAV ASSMF
EPEFS TCRAV YTKIS VLLVI LDDLY DGYGS PDEIK **LFSEA VKRWD LSLLE**
QMPDH MKICF LGLYN TVNEV AEEGR KTQGH DVLGY IRNLW EIQLA AFTRE
AEWSQ GKYVP SFDEY IENAQ VSIGV ATILL ITILF TEEDD ILSHI DYGSK
FLRLA SLTAR LANDI K**TYQE ER**AHG EVVSA IQCYM K**DRPE ITEEE ALKYV**
YGRMV NDLAE LNSEY LKSNE MPQNC KRLVF DTARV AQLFT MEGDG LTYSD
TMEIK EHIKK CLFEP AT

b

Sequence Start - End	Observed *m/z* in Dalton	Sequence (+ Modification)
116-125	1200.7446	K.RIQTLISEIK.N
117-125	1044.6407	R.IQTLISEIK.N
117-125	1045.5594	R.IQTLISEIK.N (Deamidated (NQ))
126-148	2475.1128	K.NVFNSMGDGETSPSAYDTAWVAR.I
126-148	2491.109	K.NVFNSMGDGETSPSAYDTAWVAR.I + Oxidation (M)
174-188	1854.8182	K.DGSWGEEFYFLAYDR.L
212-218	896.5091	K.GIEFFRK.Q
223-247	2865.3047	R.MDDEADNHRPSGFEIVFPAMLNEAK.S + 2 Oxidation (M)
248-265	2108.1173	**K.SLGLDLPYELPFIEQMVK.K + Oxidation (M)**
309-330	2187.0603	K.DGSFLGSPASTAAVFMQTGNTK.C
309-330	2203.0562	K.DGSFLGSPASTAAVFMQTGNTK.C + Oxidation (M)
331-340	1325.6933	K.CLEFLEFVLR.K
344-357	1701.8430	**R.NHVPSDYPLDLFER.L**
358-366	1116.6191	R.LWVVDTVER.L
379-391	1691.8073	K.DALDYVYSCWDER.G
398-412	1589.7658	**K.DSPIADIDDTAMGLR.I**
398-412	1605.7634	K.DSPIADIDDTAMGLR.I + Oxidation (M)
413-427	1723.8143	R.ILRLHGYNVSPDVLK.T
416-427	1341.7328	**R.LHGYNVSPDVLK.T**
446-455	1167.5984	R.GVTDMLNVYR.C
446-455	1183.5941	R.GVTDMLNVYR.C+ Oxidation (M)
456-471	1813.8201	R.CSQVAFPGETIMEEAK.L + Deamidated (NQ), Oxidation (M)
480-494	1705.8256	R.NALENADAFDKWAIK.K
496-506	1291.7141	**K.NIRGEVEYALK.Y**
499-511	1647.8364	R.GEVEYALKYPWHR.S
520-534	1682.8378	R.SYIGNYGPNDVWLGK.S
535-546	1507.7011	K.SLYMMPYISNEK.Y + 2 Oxidation (M)
535-552	2210.0945	K.SLYMMPYISNEKYLELAK.L + Deamidated (NQ); Oxidation (M)
535-552	2225.0266	K.SLYMMPYISNEKYLELAK.L + 2 Oxidation (M)
553-567	1814.9247	**K.LDFNSVQSLHQEEIR.E**
575-585	1272.6656	K.SSGFTELKFTR.D
636-643	949.5584	K.LFSEAVKR.W
644-657	1775.8370	R.WDLSLLEQMPDHMK.I + Deamidated (NQ); 2 Oxidation (M)
658-675	2084.0317	K.ICFLGLYNTVNEVAEEGR.K
658-676	2212.1327	K.ICFLGLYNTVNEVAEEGRK.T
676-687	1386.7639	**R.KTQGHDVLGYIR.N**
677-687	1258.6695	K.TQGHDVLGYIR.N
688-699	1461.8025	**R.NLWEIQLAAFTR.E**
700-707	934.4465	R.EAEWSQGK.Y
767-772	825.3851	K.TYQEER.A
787-798	1429.7271	K.DRPEITEEEALK.Y
787-803	2068.0552	K.DRPEITEEEALKYVYGR.M
804-817	1638.8213	R.MVNDLAELNSEYLK.S
804-817	1654.8197	R.MVNDLAELNSEYLK.S + Oxidation (M)
818-827	1280.6516	K.SNEMPQNCKR.L + Deamidated (NQ); Oxidation (M)
827-834	977.5655	K.RLVFDTAR.V
828-834	821.4633	R.LVFDTAR.V

Online Resource 3. Peptide mass fingerprint analysis of trypsin digested TPS4-HA by MALDI-TOF mass spectrometry.

a CAS_ABIBA protein sequence identified by mass fingerprint analysis of TPS-HA in the SwissProt database by Mascot Search. Peptides identified with MS are highlighted in bold. Peptide sequences additionally observed in MS/MS analysis are highlighted in bold and are underlined. The CAS_ABIBA protein was unambigously identified with a score of 191 (Expect: 4.3e-014). Protein Sequence Coverage MS: 50%. Protein Sequence Coverage MS/MS: 12%. The protein sequence of TPS4-HA is identical to CAS_ABIBA with an additional C-terminal HA-tag (YPYDVPDYA) and lacks the N-terminal predicted plastidial target sequence (highlighted in Italics, further details are given in Online Resource 2).

b Peptide sequences identified by MALDI-TOF mass spectrometry of trypsin digested TPS4-HA matching CAS_ABIBA in the SWISS-PROT database. The peptides additionally identified with MS/MS are marked in bold letters. Peptides observed in the protein sequence of CAS_ABIBA are given (Start - End of sequence, first column). The observed peak mass (*m/z*) of identified peptides is shown in the second column. Column 3 gives the protein sequence and modifications (if any).

Chapter 3: Pilot-scale cultivation of wall-deficient transgenic *Chlamydomonas reinhardtii* strains expressing recombinant proteins in the chloroplast

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Zedler, J.A.Z.*, Gangl, D.*, Guerra, T.*, Santos, E., Verdelho, V., and Robinson, C. (2016a) Pilot-scale cultivation of wall deficient transgenic *Chlamydomonas reinhardtii* strains expressing recombinant proteins in the chloroplast. *Appl Microbiol Biotechnol*. doi: 10.1007/s00253-016-7430-y

3.1. Author contribution

This publication has three first authors: myself, DG and TG. I have provided the TN72-TPS4 strain and did preliminary work for the upscaling study. Together with DG and TG I have set up the reactors with technical assistance of staff at A4F. I planned and designed the experiment together with DG taking advice from TG and CR into consideration. I produced and analysed all the data together with DG at A4F in Lisbon and later on at the University of Kent (follow up processing of samples after the experiment). I wrote parts of the materials and methods, the results and the discussion section of the manuscript and edited it. I compiled the following figures in the manuscript: Fig. 2 (with DG), Fig. 3 (with DG), Fig. 4, Fig. 5 (with DG), Fig. 6 (with DG), Fig. 7B, Fig. 8 (with DG).

3.2. Aim of this study

This study was based on the opportunity for a secondment at the industrial partner A4F in Lisbon as part of the EU FP7 project PHOTO.COMM. The company is certified to grow genetically modified organism in their contained greenhouse R&D facility in Lisbon. *Chlamydomonas reinhardtii* is a model organism, but it is not used in an industrial setting to date. To my knowledge only one study reported cultivation of *C. reinhardtii* at pilot scale (Gimpel *et al.*, 2015b). Traditionally, cell wall-deficient strains are used for transformation and there are no reports on growing transgenic wall-deficient strains although there are a lot of studies reporting recombinant protein production base on those mutant strains (see chapter 1.4). We tested if the, compared to in industrial settings usually used microalgae, fragile algae are scalable (at all) and what growth characteristics they exhibit. For this purpose we grew two transgenic strains, TN72-TPS4 (q.v. chapter 2) and TN72-CYP79A1 (q.v. chapter 5) and a CW-15 strain as a negative control in parallel in 100 L photobioreactors over a period of ten days in the green house in June 2015 at the R&D facility of A4F in Lisbon (Portugal). We monitored the culture and recombinant protein expression characteristics during the whole upscaling process.

3.3. Original research publication

Remark: Here, the pre-proof version of the manuscript is presented. The references have been added to the bibliography in chapter 7, the figures and tables numbering and position as well as headings have been changed for consistent integration into this thesis, but the content was not modified.

3.3.1. Abstract

Microalgae have emerged as potentially powerful platforms for the production of recombinant proteins and high-value products. *Chlamydomonas reinhardtii* is a potentially important host species due to the range of genetic tools that have been developed for this unicellular green alga. Transformation of the chloroplast genome offers important advantages over nuclear transformation and a wide range of recombinant proteins have now been expressed in the chloroplasts of *C. reinhardtii* strains. This is often done in cell wall-deficient mutants that are easier to transform. However, only a single study has reported growth data for *C. reinhardtii* grown at pilot scale and the growth of cell wall-deficient strains has not been reported at all. Here, we report the first pilot scale growth study for transgenic, cell wall-deficient *C. reinhardtii* strains. Strains expressing a cytochrome P450 (CYP79A1) or bifunctional diterpene synthase (*cis*-abienol synthase, TPS4) were grown for 7 days under mixotrophic conditions in a tris-acetate-phosphate medium. The strains reached dry cell weights of 0.3 g L^{-1} within 3-4 days with stable expression levels of the recombinant proteins during the whole upscaling process. The strains proved to be generally robust, despite the cell wall-deficient phenotype, but grew poorly under phototrophic conditions. The data indicate that cell wall-deficient strains may be highly amenable for transformation and suitable for commercial-scale operations under mixotrophic growth regimes.

Keywords

Chlamydomonas, cell wall-deficient, pilot scale, CYP79A1, *cis*-abienol synthase, microalgae

3.3.2. Introduction

Microalgae have considerable potential as biotechnological platforms for recombinant protein production. Many microalgae can be grown to high densities under controlled conditions (Adarme-Vega *et al.*, 2012; Borowitzka, 2013) and an attractive bonus is that sunlight can be used as an alternative or additional energy source in photobioreactors (PBRs).

Chlamydomonas reinhardtii has been a popular host strain for biotechnological applications. Genetic tools for this alga are relatively advanced and recombinant protein targets have included vaccines, antibodies, hormones and immunotoxins (Gregory *et al.*, 2012; Demurtas *et al.*, 2013; Gregory *et al.*, 2013; Jones *et al.*, 2013; Tran *et al.*, 2013a; Tran *et al.*, 2013b; Soria-Guerra *et al.*, 2014). Attempts have also been made to further develop the platform for other applications such as industrial enzymes (Rasala *et al.*, 2012; Pourmir *et al.*, 2013), biodegradable plastics (Chaogang *et al.*, 2010) and others (reviewed in (Rosales-Mendoza *et al.*, 2012; Purton *et al.*, 2013; Specht and Mayfield, 2014)).

Many studies on transgenic *C. reinhardtii* have relied on transformation of the nuclear genome for recombinant protein expression but DNA insertion into this genome is essentially random. The levels of transgene expression are unpredictable, owing to position effects, and frequently unstable because of gene silencing (Rosales-Mendoza *et al.*, 2012). Insertion of transgenes into the chloroplast genome is an attractive alternative approach, particularly since genes can be targeted to specific loci via homologous recombination and high-level and stable expression can be achieved without evidence for gene silencing (reviewed in (Purton *et al.*, 2013; Specht and Mayfield, 2014)). Genes are typically introduced by particle bombardment (Boynton *et al.*, 1988) or agitation with glass beads (Kindle *et al.*, 1991). A recently developed chloroplast transformation procedure involves the use of glass beads to introduce the gene of interest and transgenic strains are selected on the basis of restoration of photosynthetic competence. This protocol has the added advantage that the antibiotic resistance gene in the host strain is removed upon integration of the gene of interest (Economou *et al.*, 2014; Gangl *et al.*, 2015b; Zedler *et al.*, 2015). A potential disadvantage is that this mode of transformation relies on the use of cell wall-deficient host strains.

To date, transgenic *C. reinhardtii* strains have been used in a large number of studies, and this alga is rapidly gaining attention as a potential chassis for large-scale industrial production purposes. In this context, it is perhaps remarkable that only a single report has described cultivation of this alga in industrial PBRs; indeed this is all the more surprising given that many transformation procedures involve the use of cell wall-deficient strains that are inherently more fragile. Gimpel et al. described the production of bovine Milk Amyloid A in a transformed strain (possessing a normal cell wall) that expressed the protein in the chloroplast (Gimpel *et al.*, 2015b). They used 100 L hanging polybags in a greenhouse and reported expression levels of 3.28 mg L^{-1} of the target protein. Here, we describe pilot-scale culture of strains expressing a soluble protein (*cis*-abienol synthase (TPS4) from *Abies balsamea*) in the chloroplast stroma and a membrane-bound enzyme (a cytochrome P450 termed CYP79A1) in the chloroplast membrane. Bench scale studies on these strains have been recently described (Gangl *et al.*, 2015b; Zedler *et al.*, 2015) and our aim in the present study was to test whether they could be grown at scale under industrial conditions. We show that the strains grow rapidly under mixotrophic conditions and that they produce the target proteins at high levels. The strains do not, however, grow well under phototrophic conditions and we, therefore, conclude this type of strain offers high potential as a cell factory for proteins and products, but with a strong preference for mixotrophic growth regimes.

3.3.3. Materials and methods

3.3.3.1. *Chlamydomonas reinhardtii* strains and preparation of inoculum

The transgenic *Chlamydomonas reinhardtii* strains TN72-CYP79A1 and TN72-TPS4 (henceforth CYP79A1 and TPS4, respectively) have been previously characterised in (Gangl *et al.*, 2015b) and (Zedler *et al.*, 2015) respectively. The *C. reinhardtii* strain CC-400 (cw15, mt+) was used as an untransformed control (referred to as CW-15). For inoculation of the green walls pre-cultures of each strain were grown in tris-acetate-phosphate medium (TAP) (Gorman and Levine, 1965) with a modified trace element recipe (Kropat *et al.*, 2011). The cultures were bubbled with air (supplemented with 0.5 % CO_2), at 25 °C, and an illumination intensity of

170 µmol m^{-2} s^{-1}. 24 hours before inoculation cultures were supplemented with 0.1 g L^{-1} ampicillin to minimise bacterial contamination.

3.3.3.2. Pilot scale cultivation set up and sampling

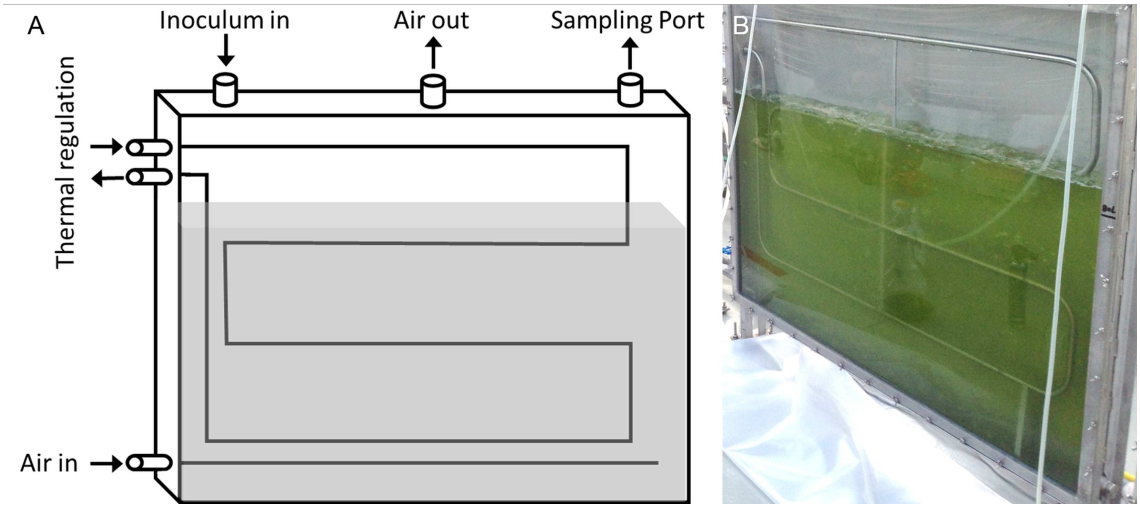

Fig. 3.1. PBRs used in this study. The Glass Green Wall PBRs used in this study were custom made from stainless steel with one side of glass and an optic path length of 9 cm. The GGW contains a lower inlet valve for air and CO_2, together with ports for sampling and thermal regulation. The GGWs were sterilised before use and used in a GM-certified outdoor facility; cultures were grown in 100 L Tris-acetate-phosphate medium as detailed in Materials and Methods. **A** is a schematic presentation of the PBR set up. **B** Picture of the PBR taken during this study.

Pilot scale cultivation was carried out in Glass Green Wall (GGW) PBRs installed inside a greenhouse of the Experimental Unit of A4F in Lisbon, Portugal. The GGWs were custom made from stainless steel with one side of glass and an optic path length of 9 cm. The maximum capacity was 120 L (Fig. 3.1). The three GGWs were filled with 100 L of TAP medium (as used for inoculation cultures). After chlorination with 50 ppm sodium hypochlorite, overnight, the reactors were boiled for 40 min to further decontaminate the culture medium. After cooling to 25 °C, the GGWs were supplemented with 0.1 g L^{-1} ampicillin to minimise bacterial contamination. The three GGWs were inoculated simultaneously with stationary phase cultures of the transformant strains (TPS4 and CYP79A1) and the CW-15 control strain on 30 June 2015 to an initial OD_{750nm} of 0.08 to 0.1. The GGWs were maintained at a temperature

of 25 °C. In addition, they were bubbled with air supplemented with 0.5 % CO_2 to maintain the pH levels of the cultures between 7 and 8 and irradiated by natural sunlight. The cultures were initially shaded with plastic sheets to allow for adjustment to the new light intensities. The sheets were removed on the second day of the experiment. Samples from the cultures were taken daily in the morning between 09.00 and 10.00. Additionally, in the afternoon between 16.00 and 17.00, the pH was measured and cell growth was analysed by cell counting and optical density measurements. The viability of the cultures was monitored by microscopy until stationary phase was reached.

3.3.3.3. Monitoring of morphology and growth

The cultures were observed daily using an Olympus BX53 upright microscope. Cell growth was analysed by cell counting with a Muse® Cell Analyser (Merck Millipore, USA). Optical density was measured at 750 nm with a GENESYS UV-Vis spectrophotometer (Thermo Scientific, USA). Dry weight was determined by filtration of 50 – 100 mL of culture through 1.2 μm glass fibre filters (Merck Millipore, USA) and subsequent drying at 180 ºC using an MS-70 moisture analyser (A&D Company, Japan).

3.3.3.4. Monitoring of culture medium parameters

The pH of the cultures was measured twice daily. Ammonium (NH_4^+) levels in the culture supernatant were analysed daily using a Nutrafin ammonium test kit (Hagen, Canada) following the manufacturer's instructions. The acetate content in the culture medium was determined using an Acetic Acid Assay Kit (Megazyme, Ireland) following the microplate assay procedure as recommended by the manufacturer.

3.3.3.5. Pigment analysis

Pigments were extracted from 5 mL of culture. The culture pellets were resuspended in 5 mL 100 % acetone. Approx. 1 mL of beads were added to each sample. The mixture was then subjected to vortexing for 1 minute and the extracts were centrifuged for 10 minutes, 3000 rpm at 4 °C. The pigment extracts were directly analysed by recording a wavelength scan using a GENESYS UV-Vis spectrophotometer (Thermo Scientific, USA) from 380 nm to 700 nm. Samples for day 7 were diluted 1:1 to obtain measurements in the linear range of absorption. For baseline correction, pure 100 % acetone was measured prior to the samples.

3.3.3.6. Homoplasmy analysis

Total genomic DNA was extracted from 20 µL to 2 mL of liquid culture following the protocol described in (Economou *et al.*, 2014). The extracts were subjected to a homoplasmy analysis by a three-primer PCR method to analyse genetic stability of the strains. Details of the experimental procedures have been described previously in (Zedler *et al.*, 2015).

3.3.3.7. Protein expression analysis: western blotting and densitometry

At each time point the same quantity of cells (2 x 10^7 cells) was spun down and resuspended in the same amount of medium. The samples were boiled with 0.1 mM DTT and 5 % SDS for 5 minutes at 95 °C for cell lysis. For each sample an equivalent of 4.8 x 10^5 cells was subjected to electrophoresis on a 12 % sodium dodecyl sulphate-polyacrylamide gel and subsequently immunoblotted with an anti-HA antibody. Details of western blotting have been previously described in (Gangl *et al.*, 2015b). Densitometry was performed using the Image Lab Software (V4.1, Bio-Rad Laboratories). A dilution series of HA-ubiquitin was loaded on the same gel as the CYP79A1 and TPS4 samples. We have assumed that transfer onto the membrane was uniform and that the affinity of the HA-antibody was equal for samples and standards

since they share the same epitope. After separation of known molar amounts of HA-ubiquitin a calibration curve was generated and the molar equivalent of CYP79A1 and TPS4 were determined.

3.3.3.8. Lab scale cultivation and generation of growth curves

CW-15, CYP79A1, TPS4 and TN72-RP strains were grown in TAP medium (Gorman and Levine, 1965) or high salt medium (HSM) (Sueoka, 1960) with a modified trace element recipe (Kropat *et al.*, 2011) at 25 °C in approximately 40 µmol m^{-2} s^{-1} constant light with shaking at 110 or 120 rpm. Samples were taken daily at the same time and the optical density was measured at 750 nm using a DU 730 UV/Vis Spectrophotometer (Beckman Coulter, UK). The experiments were performed in triplicate and the average was calculated with error bars displaying the standard error.

3.3.4. Results

We previously generated two transgenic, cell wall-deficient *C. reinhardtii* strains designated TN72-TPS4 (TPS4) and TN72-CYP79A1 (CYP79A1), which express a soluble diterpene synthase, TPS4, and a membrane-bound cytochrome P450, CYP79A1, respectively. Both strains, described in (Gangl *et al.*, 2015b) and (Zedler *et al.*, 2015) expressed the transgenes in the chloroplast following transformation using a relatively straightforward protocol (Economou *et al.*, 2014). In the present study we tested the feasibility of growing those strains in a greenhouse at pilot scale in industrial PBRs. As a control, the two transgenic strains were compared to a cell wall-deficient mutant, CW-15. However, it should be noted that only two transgenic strains could be run simultaneously in the contained PBR facilities, therefore it was not possible to grow the recipient TN-72 strain used for transformation, since it is itself transgenic (the *psbH* gene is disrupted and it expresses an *aadA* cassette). The CW-15 strain is similar, but not identical, to the TN-72 strain used for transformation (see discussion). All three strains were cultivated for seven days, in 100 L GGW PBRs, using TAP medium and natural sunlight. The PBR setup is shown in Fig. 3.1.

3.3.4.1. Growth characteristics observed in GGW PBRs

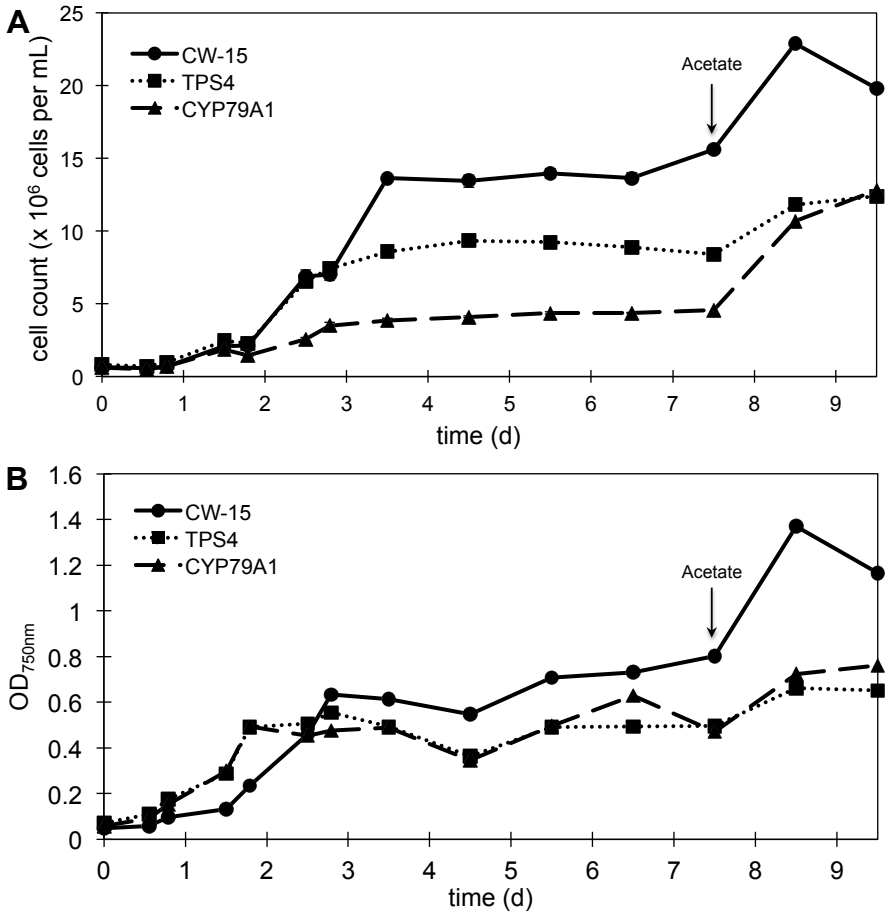

Fig. 3.2. Growth curves for transgenic strains and a cell wall-deficient control strain. The TPS4 and CYP79A1 transgenic *C. reinhardtii* cultures, plus a cell wall-deficient non-transformed strain termed CW-15, were grown in the PBRs over a 7-day period. At the indicated times, samples were removed from the PBRs and used to count cell numbers (**A**) and measure OD_{750} values (**B**). Arrows indicate points where additional acetate (50 mL glacial acetic acid) was added to the cultures

The growth monitored by cell counts and optical density measurements in the PBRs is shown in Fig. 3.2. An initial exponential growth phase was followed by a stationary phase, which all strains reached between day 3 and day 4 of the culture period. OD_{750} values reached 0.5 – 0.6 corresponding to cell counts of 4-8 x 10^6 per mL for the transformants. This stationary phase remained unchanged until the end of the growth period. In general, the CW-15 strain performed better than both transformants, reaching higher cell numbers and OD values compared to the transgenic lines. The

TPS4 strain demonstrated stronger growth compared to the CYP79A1 expressing strain as evidenced by cell counting (Fig. 3.2 A), although optical density measurement suggested a similar growth rate for both transgenic lines (Fig. 3.2 B). Dry weight measurements corresponded to the growth curves with a maximum yield of 0.438 g L^{-1} observed for the control strain CW-15 and 0.311 g L^{-1} versus 0.289 g L^{-1} for the TPS4- and CYP79A1-expressing strains, respectively (Fig. 3.3). Culture samples from all three PBRs were observed under the microscope daily to assess motility and general cell fitness. Throughout the culture period all three strains appeared 'healthy', in general terms, and were of regular size and shape (Fig. 3.4). Absorption spectra of pigment extracts from time points taken over the whole culture period showed that the pigment composition pattern was overall the same for all strains at all stages of growth (Fig. 3.5). This additionally supports our observations of an overall healthy state of the cultures.

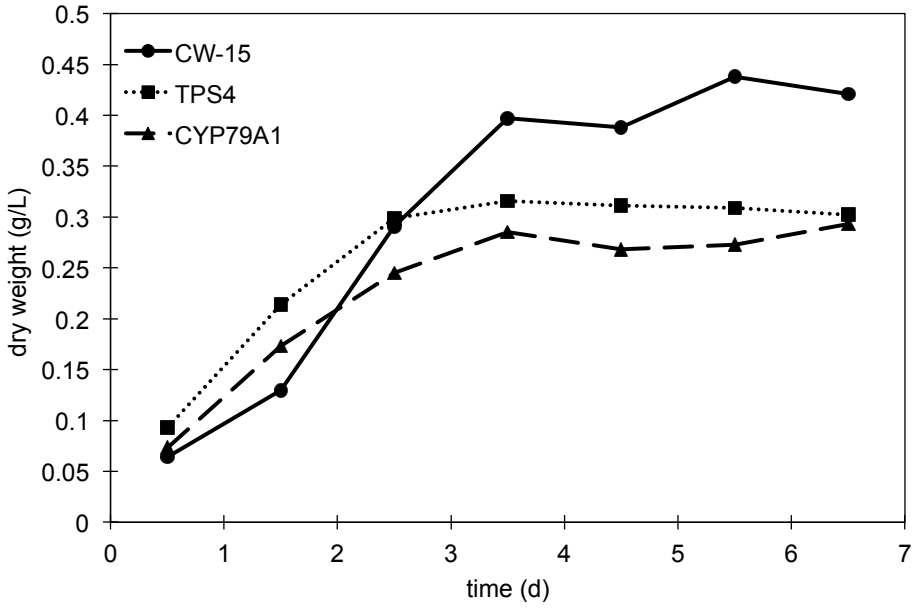

Fig. 3.3. Productivity of the cultures. The graph shows dry weights of the three PBR cultures over a seven-day period. The TPS4 and CYP79A1 transgenic algal cultures reached dry weights of ca. 0.3 g L^{-1} while the untransformed CW-15 control culture reached slightly higher values.

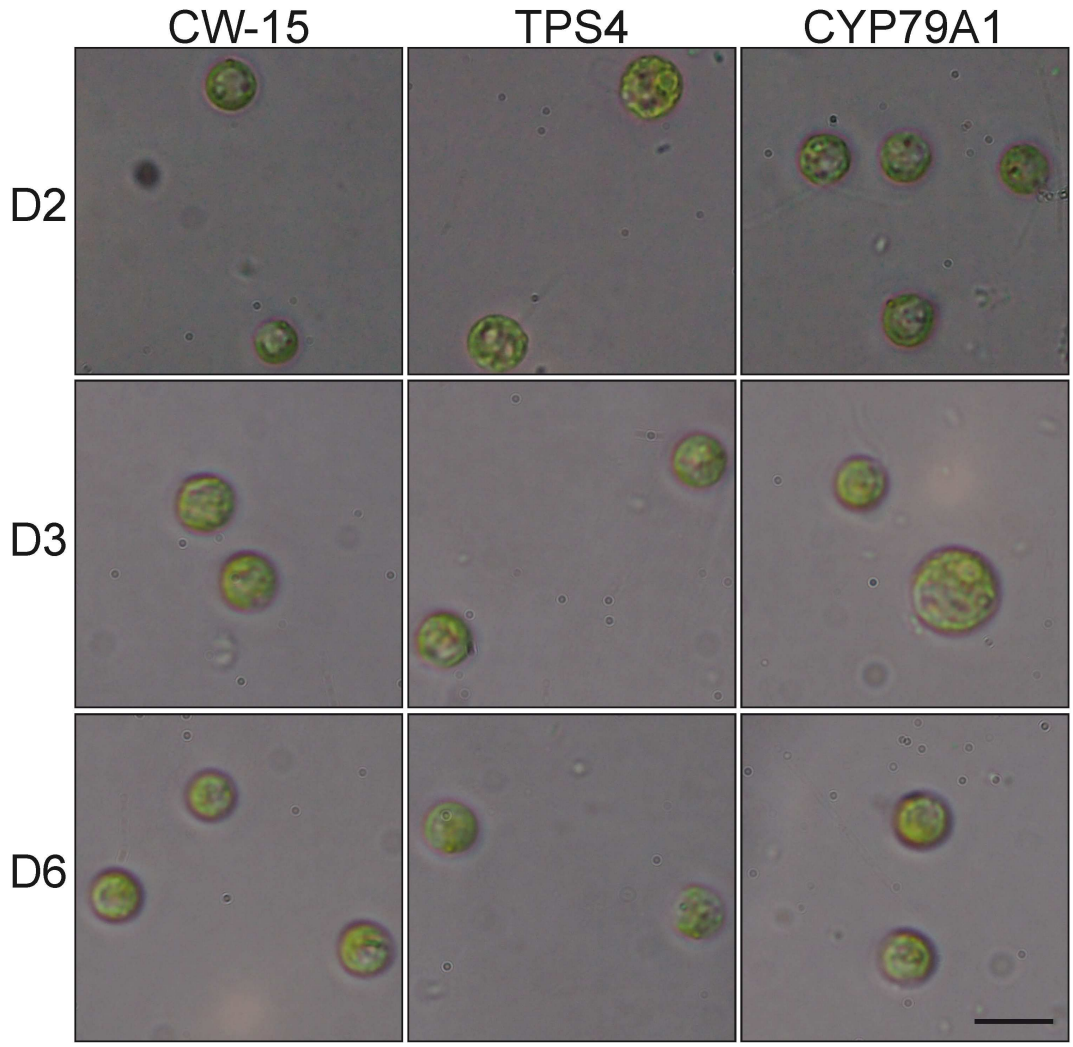

Fig. 3.4. Analysis of the *C. reinhardtii* cells by microscopy. Samples of the TPS4, CYP79A1 and CW-15 cultures were removed at days 2, 3 and 6 (D2/3/6) and analysed by microscopy as detailed in Materials and Methods. Scale bar: 10 µm.

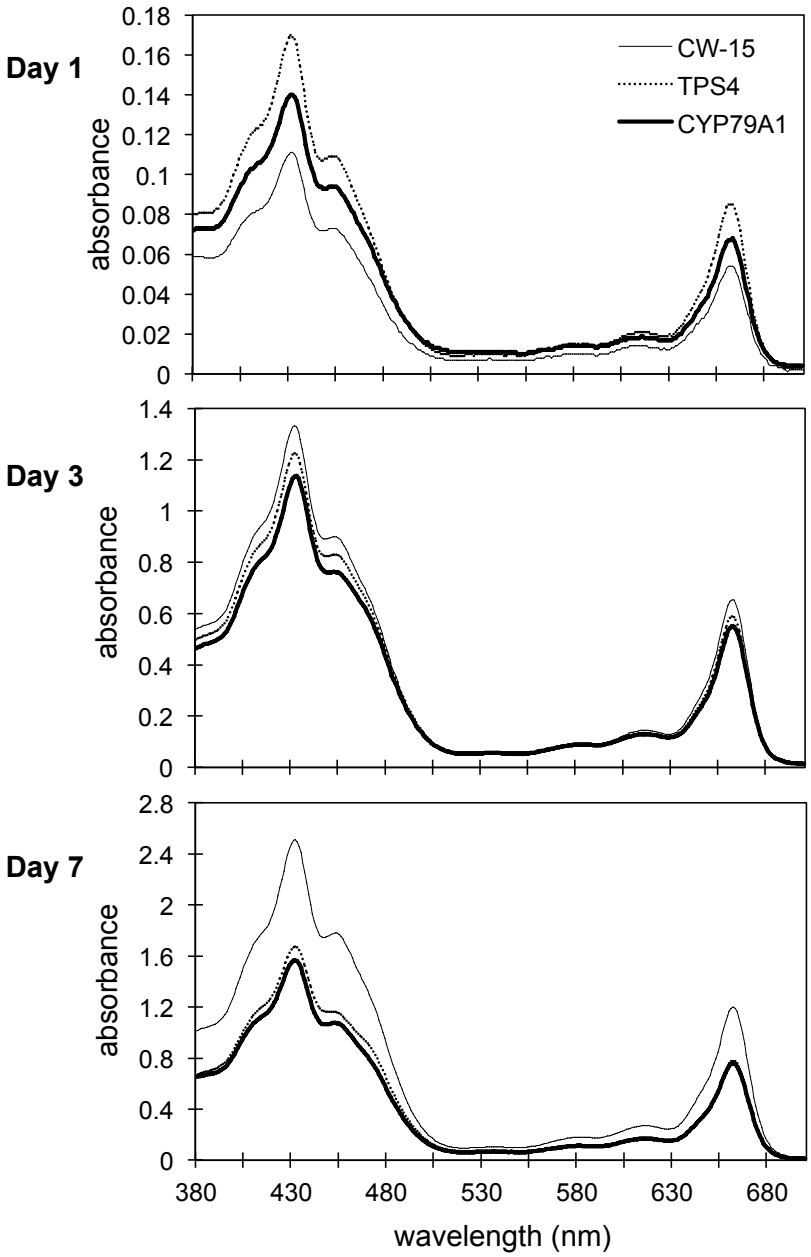

Fig. 3.5. Absorption spectra of pigment extracts from the GGW PBR cultures. Absorption spectra were recorded from pigment extracts in 100 % Acetone from 380 nm to 700 nm. The figure shows spectra recorded for the three cultures in the GGW PBRs: CW-15, TPS4 and CYP79A1 at three time points respectively: day 1, day 3 and day 7 of growth in the PBRs.

3.3.4.2. Nutrient metabolism in GGW PBRs

Daily measurements of acetate and NH_4^+ levels in the culture medium were conducted to obtain an insight into the cultures' carbon and nitrogen metabolism. NH_4^+ was steadily consumed in all of the strains over the culture period with a sudden, but

temporary, NH_4^+ increase observed on day 6 (Fig. 3.6 A). Acetate measurements revealed that essentially all of the acetate in the culture medium had been consumed after four days (Fig. 3.6 B). This corresponded with cultures reaching stationary phase around the same time. To investigate this apparent dependence on organic carbon further, we added fresh acetate to the cultures on day 8 of the experiment. As shown in Fig. 2, all three cultures resumed growth, the most dramatic effect being observed in the CYP79A1 expressing strain, which now reached similar cell numbers to TPS4.

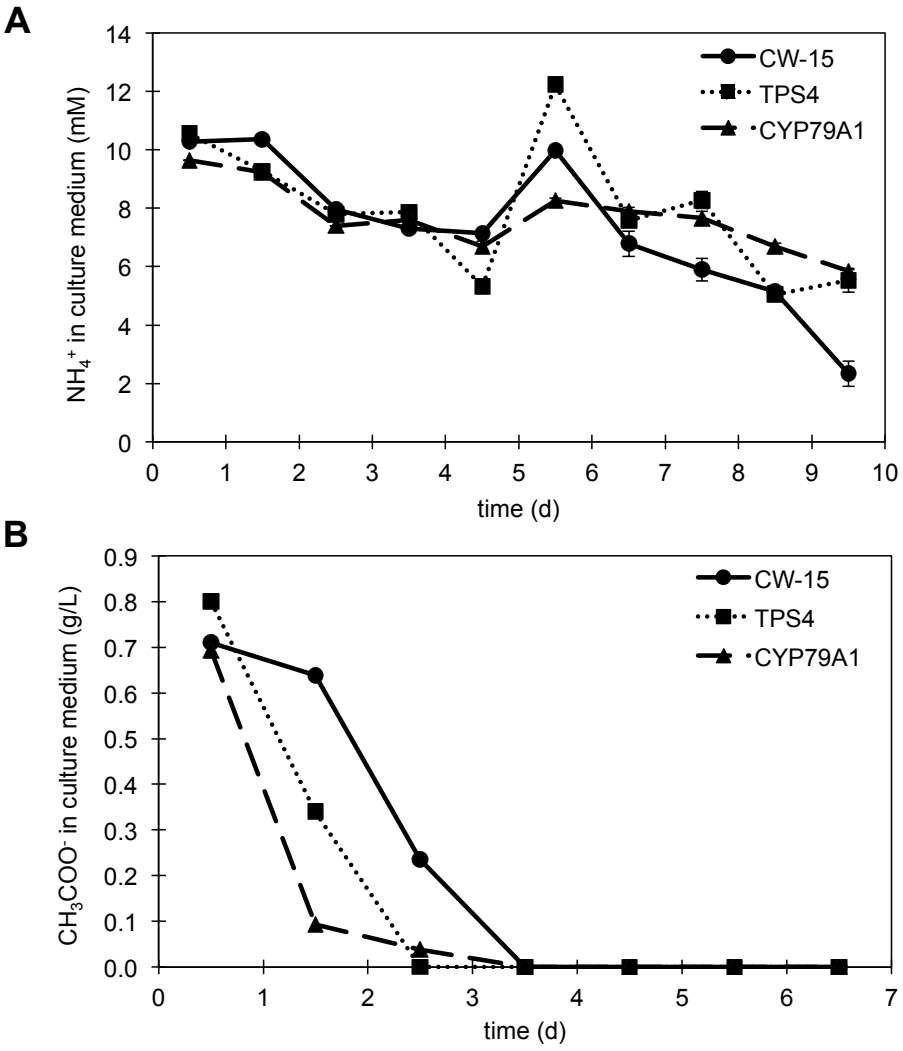

Fig. 3.6. Ammonium and acetate values in the culture medium. **A** NH_4^+ concentrations throughout the 7-day culture period. **B** Measurements of the acetate (CH_3COO^-) levels. Values were obtained as detailed in Materials and Methods.

3.3.4.3. Growth characteristics in PBRs are similar to those observed at lab scale

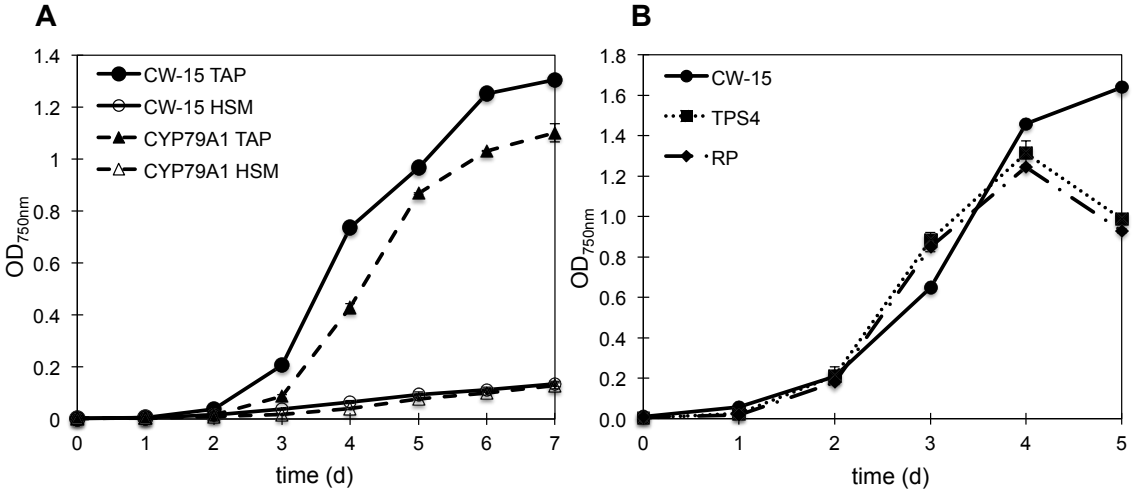

Fig. 3.7. Growth curves of wall-deficient strains at lab scale. **A** shows typical growth curves of CW-15 and CYP79A1 grown in TAP and minimal medium (HSM) in shake-flasks under laboratory conditions. At the indicated time points optical density measurements were conducted at 750 nm. The experiment was performed in triplicates and the average was calculated. Error bars display the standard error. For details see Material and methods. **B** shows the differences of growth of CW-15, TPS4 and TN72-RP (RP) at lab scale in TAP medium.

Growth curves obtained from shake-flask cultures of CW-15 and TN72-CYP79A1 in TAP and minimal medium (HSM) showed that both strains grow very rapidly in TAP medium (i.e. mixotrophically) but much slower in minimal medium (Fig. 3.7 A). This suggests that while the described transgenic strains are capable of autotrophic growth, there is a strong preference for mixotrophic growth both at lab scale and pilot scale. The overall growth pattern observed in the PBRs (Fig. 3.2) is very similar to those obtained in lab scale studies.

We have also compared the growth of the control strain CW-15 used in the PBRs with the transgenic strain TN72-RP. TN72-RP is essentially the same strain as CYP79A1 and TPS4, but lacking a gene of interest, because it was transformed with an empty pASapI vector (details are given in Zedler et al 2015). The differences in growth between TN72-RP and CW-15 observed at lab scale are very similar to the differences we found for the transformants and CW-15 in the PBRs. However the growth differences between TN72-RP and TPS4 are minimal (Fig. 3.7 B) suggesting that the

presence of the transgene does not have a marked effect on growth under these conditions.

3.3.4.4. Stable expression levels of transgenes at pilot scale

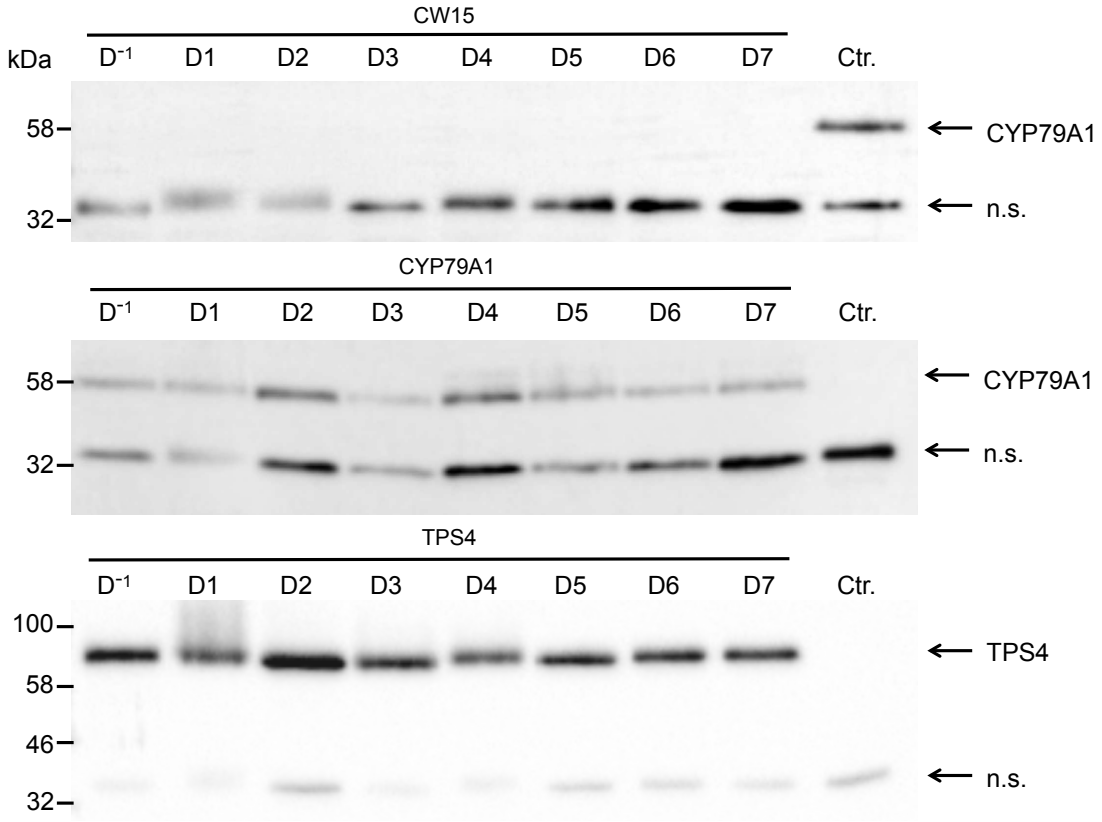

Fig. 3.8. TPS4 and CYP79A1 are produced continuously throughout the 7-day culture period. Samples were removed from the three cultures at the times indicated and analysed by immunoblotting using antibodies to the C-terminal HA tag on the TPS4 and CYP79A1 proteins. The mobilities of these proteins are indicated. N.s: protein that reacts non-specifically with the anti HA tag antiserum. Additional control (Ctr.) samples were run at the right hand side of each gel; these were samples of the CYP79A1 culture for the blot of the CW-15 culture, and samples of CW-15 culture for the blots of CYP79A1 and TPS4 cultures. In each case a sample of the inoculum was also taken 24 h before inoculation of the GGWs (Day -1 lanes).

PCR analysis of transformed culture samples at the beginning and end of the culture period confirmed that the transgenes remained stably integrated in the chloroplast genome throughout the duration of the upscaling procedure (data not

shown). This indicates that the engineered lines remain genetically stable even under the stresses of prolonged large-scale growth in an industrial PBR.

Western blotting was carried out to detect the HA-tagged TPS4 and CYP79A1 proteins, and Fig. 3.8 shows that both proteins are detected throughout the culture period. The data show the presence of the 62-kDa CYP79A1 protein and the 91-kDa TPS4 protein at all sampling points. A non-specific band of approximately 32 kDa was observed in all of the samples including the CW-15 control. This band was also detected in the previous shake-flask studies by Gangl et al. (Gangl *et al.*, 2015) and Zedler et al. (Zedler *et al.*, 2015), who showed that this band results from non-specific reaction with the anti HA tag antiserum. A calibrated immunoblot for both proteins, CYP79A1 and TPS4, with increasing loadings of a reference protein (HA-ubiquitin) allowed an approximation of protein expression levels. We estimated that the expressed proteins accumulated to levels of 6.77 mg L^{-1} (CYP79A1) and 22 mg L^{-1} (TPS4) by densitometric analysis.

3.3.5. Discussion

In this study we have grown transgenic wall-deficient *C. reinhardtii* strains at pilot scale (100 L) using GGW PBRs. The results show that these strains can grow effectively over a 10-day period and this in itself is an important finding; these are cell wall-deficient strains that are inherently less robust than true wild type strains. Prior to this study, it was not clear whether the strain would withstand the stresses involved in large-scale cultivation in a PBR with constant air/CO_2 bubbling. We now conclude that this cell wall-deficient strain can indeed be used for the production of recombinant proteins and derived products using industrial PBRs under the conditions described in this study. This is a first step to enable the transition from lab scale experiments to large scale cultivation and a basis for developing an industrial platform using this type of strain.

Gimpel et al. have previously shown that *Chlamydomonas reinhardtii* can be grown at pilot scale, however they used strains with a functional cell wall (Gimpel *et al.*, 2015b). Our data therefore represent an additional demonstration of pilot-scale *C. reinhardtii* cultivation, using a very different form of strain and under rather different

growth conditions (see below). It has been suggested that *C. reinhardtii* strains in general may be difficult to culture in industrial systems, due to low temperature tolerance (Hlavova *et al.*, 2015) and there is a clear need for pilot scale studies to assess these strains' characteristics under production conditions. Several points of interest have emerged from the present study.

3.3.5.1. Growth differences in mixotrophic and phototrophic regimes

The growth data from the GGW PBRs illustrate key points regarding these strains. Whereas the strains initially grew rapidly in TAP medium, they entered stationary phase after 3-4 days at a point that corresponded closely to depletion of the acetate in the medium. After several days in this condition the cultures again grew rapidly when more acetate was added. These data show quite clearly that while the cultures can be grown quickly in the presence of an organic carbon source under a mixotrophic regime, they grow slowly under phototrophic conditions. These findings are in reasonable agreement with results from laboratory-scale cultures. Although only a few reports have emerged using the TN72 strain, Young et al have recently demonstrated weaker growth of transformants based on this strain under phototrophic conditions compared to mixotrophic conditions (Young and Purton, 2015). We conclude that these strains are most effectively grown at pilot scale under mixotrophic growth conditions rather than phototrophic growth regimes. This has also been suggested for other green microalgae of industrial interest such as *Chlorella* sp. (Liang et al., 2009; Kong et al., 2011; Cheirsilp and Torpee, 2012) and *Scenedesmus* sp. (Girard *et al.*, 2014).

3.3.5.2. Growth differences between the transformants and the control strains

Our growth data show that the control strain CW-15 grew consistently better than the transformed strains. Here it should be noted that this is most likely due to a different genetic background rather than a transgenic phenotype. The transformants TPS4 and CYP79A1 are based on the strain TN72 (cw15, *psbH::aadA*, mt+) (described

in (Economou *et al.*, 2014)) which, although a CW-15 derivative, is not identical to the CW-15 strain used for control growth tests. This idea was supported by findings at lab scale (shown in Fig. 3.5 B) where the differences in growth of TN72-RP and CW-15 are shown. We therefore conclude that the observed differences between the transgenic strains and CW-15 are due to a slightly varied genetic background. However, at the same time we cannot exclude the possibility that the expression of the transgenes has an effect on cellular metabolism. The transformed strains express enzymes that utilise endogenous metabolites as their substrates. TPS4 converts geranylgeranyl diphosphate to *cis*-abienol and CYP79A1 catalyses the formation of *p*-hydroxyphenylacetaldoxime from tyrosine. This could potentially lead to the depletion of metabolites in the cell or to an adverse or toxic effect of the expressed compounds. However, as mentioned above we do not see any evidence of this on a lab scale. It is more likely that the different growth rates observed for the transformants is due to variabilities between the cultures in the PBRs.

3.3.5.3. Comparison of culture conditions and yields

Our findings differ from those reported by Gimpel *et al.* (2015b) in their pilot-scale study since their cultures were grown in HSM medium, without added acetate, and phototrophic growth was observed throughout the growth period. However, several points are relevant. First, they transformed a wild type (walled) *C. reinhardtii* strain rather than a cell wall-deficient strain, which may be more adept at growing phototrophically under these conditions. Secondly, the Gimpel et al. study involved growth in hanging polybags with frequent (every 30 min) bubbling with 5 % CO_2, which can boost photosynthesis to a considerable extent. In contrast, our study involved bubbling with 0.5 % CO_2 and this lower concentration may have contributed to the poor phototrophic growth performance after the acetate was depleted. On the other hand, it is notable that much more rapid growth rates are obtained under mixotrophic conditions; as an example, our transgenic cultures reached dry weights of around 0.3 g L^{-1} by day 4, whereas the cultures in the Gimpel et al. study only reached 0.267 +/- 0.011 g L^{-1} after 12 days (Gimpel *et al.*, 2015b).

We also found higher expression levels of the recombinant proteins in our cultures. Densitometry of calibrated blots gave estimates of 6.77 mg L^{-1} protein for CYP79A1 and 22 mg L^{-1} for TPS4 in these strains, compared to 2.27 +/-0.88 mg L^{-1} reported for MAA expression in the (Gimpel *et al.,* 2015b) study using transgenic strains with an intact cell wall. The reason for higher expression levels might be due to the different expression system used, to the nature of the target protein or to the culture conditions.

CYP79A1 and TPS4 are enzymes involved in the synthesis of secondary plant metabolites. The expression of many recombinant proteins in the *C. reinhardtii* chloroplast has been reported, but to our knowledge only one study investigated the expression of recombinant enzymes. Xylitol reductase from the fungus *Neurospora crassa* was expressed in the chloroplast of *C. reinhardtii* and up to 0.38 g L^{-1} xylitol was produced when xylose was supplemented to the medium (Pourmir *et al.*, 2013). In comparison, our preliminary data show that the CYP79A1 product *p*-hydroxyphenylacetaldoxime accumulates to levels of only 0.293 mg L^{-1} in the pilot scale experiment (data not shown). However, this oxime is an intermediate in a biosynthetic pathway and it may well be short-lived in this host strain.

3.3.5.4. Implications for using *Chlamydomonas* wall-deficient strains in an industrial setting

We have shown that transgenic, cell wall-deficient *C. reinhardtii* can be effectively cultured at pilot-scale in GGW PBRs, and that they can produce recombinant proteins in the chloroplast over the entire growth period. We have also shown that, while these strains are best used in mixotrophic growth regimes, they can reach high cell densities within a few days, and there is a clear potential for even higher densities by repeated addition of acetate to the culture (i.e. sustaining mixotrophy over the whole growth period). Given these strains' ability to express a cytochrome P450 and a diterpene synthase in the chloroplast at high levels, they may represent attractive platforms for the production of not only recombinant proteins but also high-value compounds such as terpenoids.

3.3.6. Acknowledgments

The authors thank all members of A4F's team that carried out the maintenance and daily operation of the GGW PBRs and Artur Włodarczyk for extraction and LC-MS analysis of the Oxime.

3.3.7. Compliance with ethical standards

Funding: The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n. 317184. This material reflects only the author's views and the Union is not liable for any use that may be made of the information contained therein.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Chapter 4: Efficient targeting of recombinant proteins to the thylakoid lumen in *Chlamydomonas reinhardtii* using a bacterial Tat signal peptide

Zedler, J.A.Z., Mullineaux, C.W., and Robinson, C. (2016b) Efficient targeting of recombinant proteins to the thylakoid lumen in *Chlamydomonas reinhardtii* using a bacterial Tat signal peptide. *Algal Res*, submitted.

4.1. Author contribution

I am the first author of this research article. The experimental design was planned by me and CR. I did all the experimental work (synthetic gene design, construct assembly, chloroplast transformation, characterisation of transformants). The confocal images were obtained by CWM from cells I prepared. CWM did the analysis of the confocal images. I drafted the manuscript and prepared all the figures, figure 4 was compiled with input from CWM and edited to its final version by CR. CR and CWM helped to write and edit the manuscript. All authors approved the manuscript before submission.

4.2. Aim of this study

To date, many studies have reported the expression of recombinant proteins in the chloroplast of *C. reinhardtii.* However, all recombinant proteins other than one study (see chapter 5) have expressed the protein in the stroma. The thylakoid lumen is a unique compartment that could be a valuable addition to the chloroplast toolkit for establishing microalgae as a cell factory. In this study the potential of utilising the intrinsic protein translocation system for lumen targeting in microalgae was investigated. Translocation to the thylakoid lumen was tested using a simple bacterial export signal peptide (TorA signal peptide). A fluorescent reporter gene, pHRed and a simple model protein of biotechnological relevance, an scFv antibody fragment, was used to test the targeting to the lumen in a first proof of concept study.

4.3. Original research publication

Remark: the manuscript is inserted as it has been submitted for publication. The references have been added to the bibliography in chapter 7, the figures and tables numbering and position as well as headings have been changed for consistent integration into this thesis, but the content was not modified.

4.3.1. Abstract

Interest in the exploitation of microalgae for biotechnological applications has increased over the last decade, and microalgae are now viewed as offering a sustainable alternative to traditionally used host chassis. A number of recombinant proteins have been expressed in genetically modified algal strains, with the green alga *Chlamydomonas reinhardtii* being a particularly popular host strain. While nuclear transformation is possible with this organism, chloroplast transformation offers more reliable expression, and several proteins have been expressed in the stroma. Here we present the first utilisation of the thylakoid lumen for recombinant protein production in microalgae. A bacterial export signal peptide was used to efficiently translocate two recombinant proteins, a fluorescent reporter protein (pHRed) and a biopharmaceutical model substrate (scFv) into the thylakoid lumen. This approach expands the algal chloroplast genetic toolkit and offers a means of expressing proteins that are difficult to express in the stroma for reasons of toxicity, stability or a requirement for disulphide bonding.

Keywords

Chlamydomonas, Thylakoid lumen, Protein Targeting, TorA signal peptide, Fluorescent Sensor, Antibody Fragment

4.3.2. Introduction

The green alga *Chlamydomonas reinhardtii* has been used as a host for the expression of a variety of recombinant proteins, and its biotechnological potential has been explored in many studies over the last decade. A number of heterologous proteins have been expressed, including vaccines, antibody fragments and terpene synthesis enzymes for recent reviews see (Almaraz-Delgado *et al.*, 2014; Gangl *et al.*, 2015a; Rasala and Mayfield, 2015). Tools for the genetic engineering of this green microalga have advanced remarkably, and it is now possible to transform both the nuclear and chloroplast genomes with reasonable efficiency. Chloroplast transformation offers the

advantage that gene integration occurs by homologous recombination at specific sites, whereas nuclear transformation is essentially random with frequent gene silencing, e.g. (Potvin and Zhang, 2010; Purton *et al.*, 2013). However, all of the chloroplast transformants reported to date have involved expression of the target protein in the stroma, with the exception of a study in which the target protein, a cytochrome P450, was targeted into a membrane (probably the thylakoid membrane) (Gangl *et al.*, 2015b).

In this study we present a novel approach to expand the genetic tool kit of the algal chloroplast involving targeting to the thylakoid lumen. The thylakoid lumen is an important compartment playing a key role in photosynthesis and energy generation in chloroplasts. However, it has a relatively small proteome (Kieselbach *et al.*, 1998) and it offers a very different environment compared to the stroma: for example, the pH is lower and the lumen is an oxidising environment that is conducive to disulphide bonding. This could have advantages for the expression of some proteins and enrich the potential of the algal chloroplast as a production platform. In chloroplasts, proteins are naturally targeted across the thylakoid membrane by the Sec or Tat pathways, and attachment of a Sec or Tat signal peptide to a heterologous protein often results in correct targeting and maturation (reviewed in (Albiniak *et al.*, 2012)). Here, we used the TorA Tat signal peptide from *Escherichia coli* which has been used to direct the export of biotechnologically relevant proteins to the periplasm in *E. coli* (reviewed in (Walker *et al.*, 2015)) as a targeting peptide to translocate recombinant proteins into the thylakoid lumen of the *C. reinhardtii* chloroplast. We show that the Tat signal peptide can target and translocate both a fluorescent reporter protein, pHRed, and a biopharmaceutical (scFv antibody fragment) into the thylakoid lumen of *C. reinhardtii*.

4.3.3. Material and methods

4.3.3.1. Plasmid construction

The sequence for the pHRed fluorescent protein was obtained from the plasmid GW1-pHRed (ORF3, addgene plasmid 31473) (Tantama *et al.*, 2011). The sequence for scFvIL1B (scFv) was obtained from the plasmid pYU49 (Matos *et al.*, 2014). An HA-tag (amino acid (AA) sequence YPYDVPDYA) was added at the C-terminus of every synthetic gene for detection by western blotting. Two constructs were made for each

protein, pHRed and scFv respectively: one stroma control (sequence encoding mature protein only) and one with a bacterial Tat export signal peptide from TMO reductase (AA sequence: NNNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATAAQAA inserted after the methionine start codon and the first amino acid of the mature protein sequence) referred to as "TorA signal peptide" (Méjean *et al.*, 1994). All genes were codon-optimised for chloroplast expression in *C. reinhardtii* using the software 'Codon Usage Optimizer' (codonusageoptimizer.org/download/). The synthetic genes were custom synthesised by GenScript (USA). All constructs made for this study were based on the vectors pASapI (Economou *et al.*, 2014) and pSRSapI (Young and Purton, 2014) for chloroplast expression in *C. reinhardtii*. An overview of the constructs made for this study is given in Table 4.1. Plasmid pJZ19 was assembled with the Gibson assembly method (Gibson *et al.*, 2009). All other constructs were made by cutting with the restriction enzymes *SapI* and *SphI* (NEB) and subsequent ligation into pASapI/pSRSapI. All constructs were sequenced to confirm the correct nucleotide sequence of the synthetic gene.

Table 4.1. Overview of constructs described in this study. All constructs were made using the transformation vectors pASapI (*atpA* 5'UTR) (Economou *et al.*, 2014) or pSRSapI (*psaA* 5'UTR) (Young and Purton, 2014) for integration downstream of *psbH* in the chloroplast genome. The predicted location of the protein in the chloroplast (*) is based on the presence or absence of a TorA signal peptide in the presequence of the protein.

Plasmid	Synthetic gene	Encoded protein	Expected MW (kDa)	Predicted location*
pJZ21	*psaA 5'UTR-scFv1L1b-HA*	scFv-HA Single-chain Fv (recombinant antibody fragment, C-terminal HA-tag) against interleukin 1β (Wilkinson *et al.*, 2009; Matos *et al.*, 2014)	27.8	Stroma
pJZ20	*atpA 5'UTR-scFv1L1b-HA*			
pJZ23	*psaA 5'UTR-TorAsp-scFv1L1b-HA*	TorA-scFv-HA (scFv-HA with N-terminal TorA leader peptide)	Pre sequence: 32.3 Mature size: 27.8	Thylakoid lumen
pJZ22	*atpA 5'UTR-TorA-scFv1L1b-HA*			
pJZ25	*psaA 5'UTR-pHRed-HA*	pHRed-HA (Tantama *et al.*, 2011)	27.3	Stroma
pJZ26	*psaA 5'UTR-torA-pHRed-HA*	TorA-pHRed-HA (pHRed-HA with N-terminal TorA leader peptide)	Pre sequence: 31.7 Mature size: 27.3	Thylakoid lumen
pJZ19	*atpA 5'UTR-torA-pHRed-HA*			

4.3.3.2. Cultivation and chloroplast transformation of *C. reinhardtii*

All *C. reinhardtii* strains were cultivated in TAP medium using the recipe as described by Gorman and Levine (Gorman and Levine, 1965) with modified trace element solutions (Kropat *et al.*, 2011). For selection of chloroplast transformants and confocal imaging (see section 2.6. for details) HSM medium (Sueoka, 1960) with the modified trace element solutions (Kropat *et al*., 2011) was used. The protocol for chloroplast transformation was used as described in Economou et al. (Economou *et al.*, 2014) using the strain TN72 (cw15, *psbH::aadA,* mt+) as a recipient. Further details of the cell line generation were as previously described in Zedler *et al.* (2015). Other than the cell lines generated in this study by transformation with the plasmids as shown in Table 4.1, a strain with a restored functional *psbH* gene was made by transforming pSRSapI (Young and Purton, 2014) without any synthetic gene integrated into TN72. This strain was named TN72-RP* and served as a negative control for transformants based on the pSRSapI vector. The strain TN72-RP (TN72 transformed with pASapI) (Zedler *et al*., 2015) was used as a negative control for pASapI based transformants.

4.3.3.3. Homoplasmy analysis of transformants by PCR

A Chelex-100 resin (Bio-Rad) was used to extract total genomic DNA from *C. reinhardtii* using a protocol described elsewhere (Economou *et al.*, 2014). Transformants generated with the pASapI vector were analysed by PCR as described in Zedler *et al.* (2015). The same protocol was used for transformants generated with pSRSapI-based constructs. The primers FLANK1, rbcL.F (both previously described (Zedler *et al*., 2015)) were used in conjunction with the primer psaA.R (5'-GGATTTCTCCTTATAATAAC-3') in a standard PCR protocol with an annealing temperature of 54 °C. Sequences for the primer design were kindly provided by Saul Purton (University College London, UK).

4.3.3.4. Cell lysis, SDS-PAGE and western blotting

Crude cell lysates for protein expression analysis were prepared from *C. reinhardtii* cells that were grown in 6 well plates in TAP medium at 25 °C, 110 rpm shaking and approx. 50 µE. A volume of cells equivalent to 0.5 mL of a culture with an optical density of OD_{750}=1 measured on a DU 730 UV/Vis Spectrophotometer (Beckman Coulter) were harvested from each sample and resuspended in 0.1 mL 10 mM Tris-HCl (pH 8.0). 0.025 mL 5x SDS protein gel loading buffer (containing β-mercaptoethanol as a reducing agent) were added to the samples and then boiled at 95 °C for 5 minutes. The crude lysates were separated and analysed by SDS-PAGE on a 15 % sodium dodecyl sulphate-polyacrylamide gel and immunoblotted. An HA-antibody (Sigma-Aldrich) was used to detect the target protein and an AtpB-antibody (Agrisera, Sweden) as a loading control.

4.3.3.5. Chloroplast isolation and fractionation

Chloroplasts were isolated from 1 L liquid cultures that were grown in TAP medium to mid log phase at 25 °C, 120 rpm shaking and constantly illuminated with approx. 50µE. The protocol described by Mason et al. (Mason *et al.*, 2006) was used for chloroplast isolation. After washing the isolated chloroplasts in 0.1 % BSA isolation buffer, the chloroplasts were directly resuspended in hypotonic lysis buffer for fractionation into stroma and thylakoids (a membrane fraction also including the chloroplast envelope membranes) as described in Balczun et al (Balczun *et al.*, 2006). The lysate loaded on 1 M Sucrose cushions was centrifuged in a Beckman TL-100 ultracentrifuge at 95 000 rpm, 4 °C for two hours using a TLA100.3 rotor (Beckman). The thylakoid fraction was then resuspended in 1x lysis buffer (Balczun *et al*., 2006). Samples were boiled at 50 °C for 10 minutes and subjected to SDS-PAGE and Western Blotting as described in Section 2.4. The samples were immunoblotted with the HA-antibody and with a PsbO antibody kindly provided by Saul Purton (University College London, UK) as a control for the fractionation.

4.3.3.6. Confocal imaging of *Chlamydomonas* cells

C. reinhardtii cells were taken from liquid cultures in HSM medium, spotted onto glass microscope slides and covered with glass cover-slips. Cells were imaged using a Leica TCS SP5 laser-scanning confocal microscope, using a 63x oil-immersion objective (NA 1.4) and excitation with a 561 nm laser line. Fluorescence emission was detected simultaneously at 600-620 nm for pHRed and 670-720 nm for chlorophyll. The confocal pinhole was set to give a *z*-axis resolution of about 1.5 μm. Images were recorded with scanning at 400 Hz, with each line generated by an average from 6 scans. Quantitative image analysis was with Image J software, with statistics from SigmaPlot 13.0.

4.3.4. Results and discussion

4.3.4.1. pHRed and an scFv are robustly expressed in the chloroplast

The aim of this study was to test for targeting of a reporter protein, pHRed, and a biotechnologically relevant protein (an scFv) to the thylakoid lumen by the Tat pathway. For comparisons of expression levels, and for control purposes, we also expressed the mature-size pHRed protein in the stroma. Screening relied on the restoration of phototrophic growth after transformation, as homologous recombination restores the intactness of the *psbH* gene (see (Economou *et al.*, 2014) for details). One series of constructs was cloned into the plasmid pASapI, which uses the *atpA* promoter, and a second series of transformations was carried out using constructs based on the plasmid pSRSapI, which uses the *psaA* promoter. *C. reinhardtii* chloroplast transformants expressing the constructs detailed in Table 4.1 were successfully generated using the recipient strain TN72 as detailed in Economou et al. (2014). Homoplasmy analysis by PCR confirmed that all strains were homoplasmic ensuring stable integration of the gene into the chloroplast genome (Fig. 4.1).

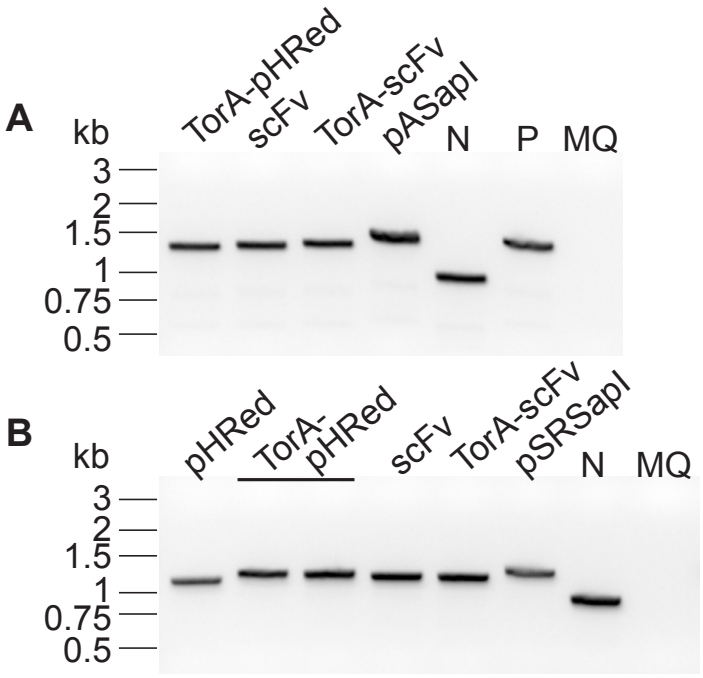

Fig. 4.1. Homoplasmy analysis of transformants expressing pHRed and scFv constructs. PCR for homoplasmy analysis was carried out on the TN72 strains transformed with constructs based on pASapI (**A**) and pSRSapI (**B**). All lanes indicate the construct that was used for transformation (details shown in Table 4.1) to generate the respective strain. pJZ26a/26b represent two separate transformants expressing TorA-pHRed. N denotes the strain TN72, used as a negative control for the PCR reaction (i.e. with no gene integrated), P denotes a positive control (i.e. gene has been integrated). In the lanes 'MQ' water was used instead of a DNA template as a negative control for the PCR reaction.

Further analysis of the transformants by SDS-PAGE and western blotting of crude cell lysates showed that the cells were expressing the respective protein, i.e. scFv or pHRed, in the chloroplast at stable levels (Fig. 4.2). Fig. 4.2 A shows (from left to right) blots of stromal pHRed and TorA-pHRed (in 2 different transformants), expressed from the same *psaA* promoter. The protein is clearly detected as a band of ca. 27 kDa and the levels of the stromal and lumen-targeted versions are reasonably similar. The next lane shows that lower TorA-pHRed levels were obtained when expressed from the *atpA* promoter, and the band is absent from the control strains transformed with empty pSRSapI or pASapI vector (RP*, RP).

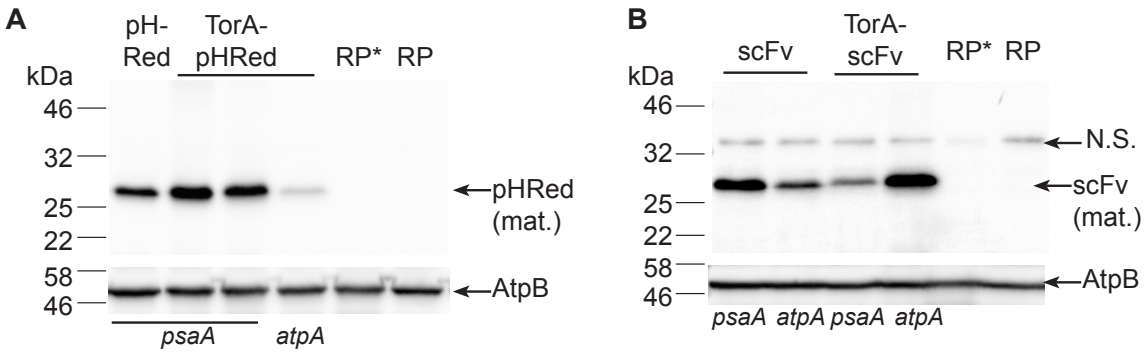

Fig. 4.2. Expression of pHRed and scFv with and without TorA signal peptide in the chloroplast of *C. reinhardtii*. **A** shows an immunoblot of cell lysates from strain TN72 transformed with the following constructs from left to right: pJZ25 (pHRed); JZ26a, pJZ26b, pJZ19 (TorA-pHRed); pSRSapI (RP*); pASapI (RP). The blot was probed with antibodies to the HA tag on the C-termini of the target proteins, and the arrow indicates the mature protein size of pHRed. **B** shows a blot of cell lysates from TorA-scFv and mature-size scFv constructs. The TN72 transformant strains from left to right are: pJZ21, pJZ20 (scFv); pJZ23, pJZ22 (TorA-scFv); pSRSapI (RP*), pASapI (RP). The mature-size scFv protein is marked with an arrow. N.S. indicates a non-specifically reacting band that has been previously observed (Zedler *et al*., 2015). In both A and B the promoter used to drive expression is shown below the blot. The blots below the anti-HA blot were probed with an anti-AtpB antibody showing approximately equal loading of lysates in all lanes; the AtpB protein is marked with an arrow.

Fig. 4.2 B shows blots of transformants expressing the 28-kDa scFv in the stroma from the *psaA* or *atpA* promoter, with slightly higher levels detected in the former. Slightly surprisingly, expression of TorA-scFv yields somewhat different results, with protein levels higher when expressed from the *atpA* promoter.

In both the A and B panels, the blots were reprobed using antibodies to AtpB as loading controls. It is also notable that in Fig. 4.2 B we detect a band of about 34 kDa which has previously been shown to stem from non-specific reaction of an unknown endogenous protein with the anti-HA antibodies (Zedler *et al*., 2015).

4.3.4.2. The precursor proteins TorA-pHRed and TorA-scFv are processed efficiently

Interestingly, in the strains expressing TorA-pHRed and TorA-scFv (Fig. 4.2), only the mature protein sizes 27/28 kDa were detected, with the same molecular weights as the stromal versions. No precursor protein, which would be expected to be around 32 kDa for both proteins, was observed in our experiments. Lumen-targeted precursor proteins are processed to the mature size after translocation by a lumen-facing processing peptidase (Albiniak *et al.*, 2012), so this provides preliminary evidence that both proteins may be targeted to the thylakoid lumen and processed to the mature size.

4.3.4.3. The TorA export signal peptide enables protein translocation to the thylakoid lumen in the algal chloroplast

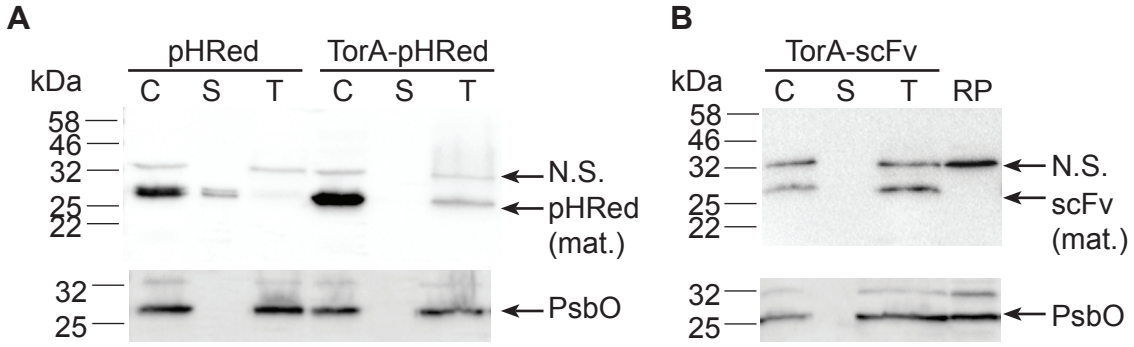

Fig. 4.3. Fractionation of chloroplasts into stroma and thylakoids (including envelope membranes). Whole chloroplast lysates (C), the stroma fraction (S) and the thylakoid fraction (T) are shown. **A** shows an Anti-HA immunoblot of chloroplast fractions from strain TN72 transformed with pJZ25 (pHRed) and pJZ26 (TorA-pHRed). **B** shows the fractions of TN72 transformed with pJZ23 (TorA-scFv). RP denotes the negative control showing that the band indicated with N.S. is a non-specific reacting band present in the negative control. A second immunoblot probed with an PsbO antibody, shown in A and B respectively, serves as a control for the fractionation. The PsbO protein is denoted with an arrow.

Although the absence of the precursor protein is indirect evidence of targeting to the lumen, fractionation studies were deemed essential to confirm this point, and chloroplasts were therefore isolated and fractionated into stroma and thylakoids by hypotonic lysis. The results of the fractionation are shown in Fig. 4.3, with the target proteins again detected by immunoblotting with antibodies to their C-terminal HA tags. Both TorA-pHRed (Fig. 4.3 A) and TorA-scFv (Fig. 4.3 B) were translocated into the thylakoids, with the mature-size proteins ('mat') clearly detected in the thylakoid fraction (T) but not the stroma (S). The stromal pHRed is detected in the stroma as expected (Fig. 4.3 A) as was the stromal scFv (data not shown). The 34 kDa band from non-specific binding of the anti-HA antibody is also apparent.

The fractions were also probed with a PsbO antibody as a control for the fractionation; PsbO is a well-known lumenal protein that forms part of the photosystem II oxygen-evolving complex. This control confirms that the targeted proteins are indeed in the thylakoid fraction. From these results, it is apparent that a bacterial Tat export signal peptide is suitable for translocation of proteins to the thylakoid lumen in *C. reinhardtii*.

4.3.4.4. TorA-pHRed is specifically targeted to the thylakoids

Representative confocal images of the strains expressing the fluorescent reporter gene *pHRed* are shown in Fig. 4.4. All images were recorded with 561 nm excitation and emission at 600-620 nm, a combination which proved to give the most selective visualisation of pHRed relative to the background fluorescence from the photosynthetic pigments. However, even at these wavelengths there was significant non-pHRed fluorescence from the thylakoid membranes (see right-hand panels of Fig. 4.4, showing the control strain), which complicates analysis of the distribution of pHRed. For quantitative comparison of fluorescence yields we manually selected either the whole cell or the pyrenoid region in the chloroplast (see Fig. 4.4) and measured the mean fluorescence in these regions, a procedure which automatically corrects for differences in cell size. For cells expressing stromal pHRed, mean fluorescence at 600-620 nm was 36 % higher than in the control strain ($n = 20$, $p = 0.00013$ from a Student's t-test). The difference was even more pronounced when fluorescence was measured only from the pyrenoid region, an area of the chloroplast stroma where there is an optically-resolvable

gap between the thylakoid membranes so that the background fluorescence from the photosynthetic pigments is lower in this region (see Fig. 4.4). In the pyrenoid region, 600-620 nm mean fluorescence from cells with stromal pHRed was 65 % higher than in the control strain (n = 20, p = 0.000087), and most cells with stromal pHRed showed an obvious fluorescence signal from the pyrenoid that was absent from the other strains (Fig. 4.4).

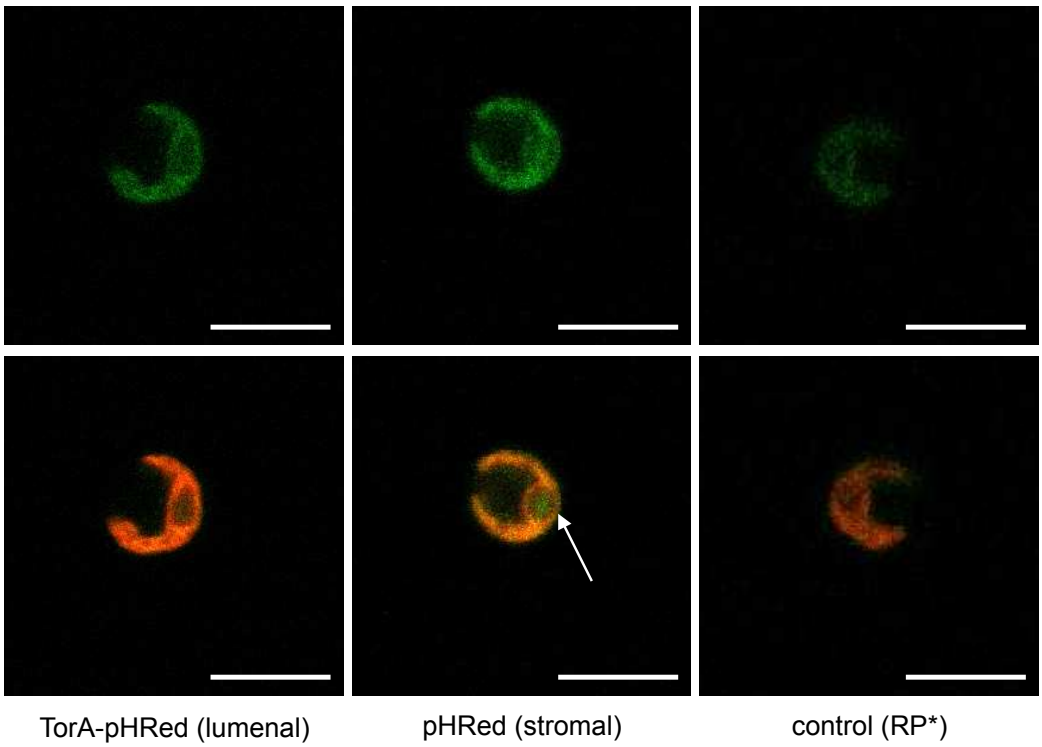

Fig. 4.4. Representative confocal fluorescence images of *C. reinhardtii* cells expressing pHRed and TorA-pHRed, with cells having the empty transformation plasmid integrated (RP*) as a control. Top: images of fluorescence in the pHRed region 600-620 nm, shown in green. Bottom: the same images merged with chlorophyll fluorescence at 670-720 nm, shown in red. The white arrow highlights stromal pHRed fluorescence from the pyrenoid region. Scale bar: 10 μm.

Cells expressing TorA-pHRed) showed mean cell fluorescence only marginally (6 %) higher than the control strain, without compelling statistical significance for a difference (n = 20, p = 0.24). Western blots indicate that TorA-pHRed protein is present at similar levels to stromal pHRed (Fig. 4.2), so it appears that fluorescence from lumenal pHRed must be somewhat quenched relative to stromal pHRed. This quenching cannot be a simple consequence of pH difference, since pHRed should show enhanced fluorescence with excitation at 561 nm at the lower pH expected in the thylakoid lumen

(Tantama *et al.*, 2011). Our fluorescence images confirm different distributions of stromal and lumenal pHRed demonstrated by the fractionation experiments (Fig. 4.3A). Lumenal pHRed appeared largely absent from the pyrenoid region of the stroma, since fluorescence in this region was 28 % lower than in the strain expressing stromal pHRed (n = 20, p = 0.00038) and only marginally higher than in the control strain (18 % higher, n = 20, p = 0.026).

4.3.4.5. Potential of lumen targeting in microalgae for biotechnology

The Tat machinery is specialised for the translocation of fully folded proteins and it has previously been shown that the bacterial Tat system has quality control (proofreading) capabilities, such that *correctly*-folded proteins are preferentially transported (reviewed in (Robinson *et al.*, 2011)). Correct protein folding is highly advantageous for recombinant protein production, and if the thylakoid Tat system has similar properties, the lumen may therefore offer certain advantages over the stroma; with the transported proteins exhibiting high folding fidelity. The lumen may also represent a beneficial environment for the production of disulphide-bonded proteins. In tobacco chloroplasts, disulphide bond formation tested with a recombinant protein (alkaline phosphatase) was reported to be more efficient in the thylakoid lumen than in the stroma (Bally *et al.*, 2008). This supports the idea of the thylakoid lumen as a novel compartment for recombinant protein production. Finally, a number of potential target proteins may be toxic in the stroma, or may catalyse unwanted metabolic processes, and the lumen may offer a 'safe haven' for such proteins.

4.3.5. Conclusion

We have shown that a bacterial Tat export signal peptide is capable of directing the translocation of model and biotechnologically relevant recombinant proteins into the thylakoid lumen of the *C. reinhardtii* chloroplast. The thylakoid lumen may therefore provide a protective environment for delicate proteins that require tight folding, especially for proteins that are potentially toxic or which are more stable at a lower pH.

This process thus represents an addition to the 'algal chloroplast toolkit' with potential for enhancing the competitiveness of microalgae as production platforms.

4.3.6. Research contribution

JAZZ designed the experiments, acquired and analysed the data, drafted and approved the manuscript. CWM acquired the confocal images, analysed the images, wrote and approved the manuscript. CR designed the experiments together with JAZZ, approved and edited the manuscript.

4.3.7. Acknowledgments

The authors thank Tchern Lenn for assistance with the confocal imaging. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n. 317184. This material reflects only the authors' views and the Union is not liable for any use that may be made of the information contained therein.

Chapter 5: Expression and membrane-targeting of an active plant cytochrome P450 in the chloroplast of the green alga *Chlamydomonas reinhardtii*

Gangl, D., Zedler, J.A.Z., Włodarczyk, A., Jensen, P.E., Purton, S., and Robinson, C. (2015) Expression and membrane-targeting of an active plant cytochrome P450 in the chloroplast of the green alga *Chlamydomonas reinhardtii*. *Phytochemistry* **110**: 22–28.

5.1. Author contribution

I am the second author of this research study. Together with DG I set up the cultivation and transformation unit in the lab of CR at the University of Kent that allowed this work to be carried out. My experimental contribution to this study was to generate the strain TN72-RP that was used as a control for this study. This consisted of chloroplast transformation, screening for positive transformants and confirming the strain identity by PCR. I discussed and analysed the research data with the other authors of this manuscript and helped with editing and proof reading of the manuscript.

5.2. Aims of this study

A more recent aspect of microalgae exploitation is the synthesis of high-value compounds such as terpenes rather than commodities by genetically modified algae. The synthesis of these terpenes in plants typically requires multiple enzymes and complex pathways in the host plant cell. Transferring those pathways into microalgae is therefore very challenging: (1) the pathway has to be well characterised to provide the needed background information, (2) usually several genes have to be inserted into the microalgal genome, (3) those genes have to be expressed at decent levels, (4) the enzyme must be in an active form, and (5) the expressed enzymes have to be able to work together, i.e. "form a metabolic pathway" to produce the desired molecule. Terpene synthesis will typically involve several cytochromes P450 (Lassen *et al.*, 2014b). In plants, these are located in the ER. It has previously been shown that it is possible to relocate a cytochrome P450 from the ER into the chloroplast and essentially scavenge reducing power from photosynthesis to drive the P450 (Nielsen *et al.*, 2013; Lassen *et al.*, 2014a; Wlodarczyk *et al.*, 2016).

This study is a proof of concept investigating if P450s, that are essential components of terpene synthesis, can be relocated to the chloroplast of a eukaryotic microalga, *C. reinhardtii*, and be expressed in an active form. A simple model cytochrome P450 from the dhurrin pathway, CYP79A1 (Sibbesen *et al.*, 1995) was expressed chosen for this proof of concept study and the significance for high-value compound production in microalgae is discussed.

5.3. Original research publication

Remark: Here, the pre-proof version of the manuscript is presented. The references have been added to the bibliography in chapter 7, the figures and tables numbering and position as well as headings have been changed for consistent integration into this thesis, but the content was not modified.

5.3.1. Abstract

The unicellular green alga *Chlamydomonas reinhardtii* has potential as a cell factory for the production of recombinant proteins and other compounds, but mainstream adoption has been hindered by a scarcity of genetic tools and a need to identify products that can be generated in a cost-effective manner. A promising strategy is to use algal chloroplasts as a site for synthesis of high-value bioactive compounds such as diterpenoids since these are derived from metabolic building blocks that occur naturally within the organelle. However, synthesis of these complex plant metabolites requires the introduction of membrane-associated enzymes including cytochrome P450 enzymes (P450s). Here, we show that a gene (*CYP79A1*) encoding a model P450 can be introduced into the *C. reinhardtii* chloroplast genome using a simple trans- formation system. The gene is stably expressed and the P450 is efficiently targeted into chloroplast membranes by means of its endogenous N-terminal anchor domain, where it is active and accounts for 0.4 % of total cell protein. These results provide proof of concept for the introduction of diterpenoid synthesis pathways into the chloroplast of *C. reinhardtii.*

Keywords

Chlamydomonas, Chloroplast transformation, Cytochrome P450, Diterpenoids, Thylakoid membrane

5.3.2. Introduction

The green alga *Chlamydomonas reinhardtii* has served as an important model organism for studies on photosynthesis, chloroplast biology and cell physiology. However, this freshwater alga has also evoked interest as a production platform for recombinant proteins and other products; heterologous genes can be expressed in both the nuclear and chloroplast genomes (Purton *et al.*, 2013; Rasala *et al.*, 2014), and the organism grows rapidly and is relatively inexpensive to culture. These factors raise the possibility that cultures can be grown in large-scale photobioreactors, which significantly reduces the risk of contamination and the escape of genetic modified strains to the environment, and makes rapid scale-up possible. In addition, green algae fall into the GRAS (generally regarded as safe) category, potentially eliminating some downstream processing steps associated with transgenically produced therapeutics (Specht *et al.*, 2010; Rasala and Mayfield, 2011).

The chloroplast of *C. reinhardtii* has been a preferred site for the expression of commercially attractive products since it offers a number of advantages compared to the transformation of the nuclear genome. In the chloroplast, high gene expression levels can be achieved and it is possible to target transgenes to defined sites using homologous recombination. In contrast, genes introduced into the nuclear genome integrate randomly and are therefore prone to position effects and RNA silencing (Day and Goldschmidt-Clermont, 2011).

In recent years several advances have been made in the field of *Chlamydomonas* chloroplast transformation (Purton *et al.*, 2013). Nevertheless, most transformation protocols still rely on antibiotic resistance for selection. A commonly used selectable marker is the *aadA* gene (bacterial aminoglycoside 3'-adenyl transferase), which confers resistance to spectinomycin and streptomycin (Goldschmidt-Clermont, 1991). Alternatively, cloned variants of the endogenous ribosomal RNA genes *rrnS* and *rrnL* can be used as markers. These carry point mutations rendering the ribosomes insensitive to certain damaging antibiotics (Newman *et al.*, 1990). However, such antibiotic selection is prone to high rates of false positives, and in the latter case results in a mutant ribosome in which translation of a highly expressed transgene is potentially compromised. Furthermore, ensuring that transgenic lines are homoplasmic (i.e., that all ~80 copies of the polyploid chloroplast genome carry the introduced transgene) requires

multiple rounds of single colony isolation under antibiotic selection. A more elegant method is to rely on the restoration of photosynthetic growth using a *Chlamydomonas* chloroplast mutant defective in an essential photosynthesis gene such as *atpB*, or *psbH* (Boynton *et al.*, 1988; Economou *et al.*, 2014). Importantly, the gene of interest remains the only segment of foreign DNA in the transformed genome. A similar strategy has been described earlier by (Cheng *et al.*, 2005).

To-date, over 50 different recombinant proteins have been synthesised successfully in the *C. reinhardtii* chloroplast, including markers, reporters, enzymes, and proteins of therapeutic value such as antibodies, hormones and vaccines (Purton *et al*., 2013). A fully functional human IgG1 monoclonal antibody against anthrax protective antigen 83 was expressed from two separate genes and assembled into a fully active antibody (Tran *et al.*, 2009). In addition, immunotoxins for cancer therapeutics (Tran *et al.*, 2013b) as well as vaccine candidates against malaria or foot-and-mouth-disease virus have been produced in the chloroplast (Gregory *et al.*, 2013; Jones *et al.*, 2013). However, most of the recombinant proteins produced, have been soluble proteins, and furthermore have generally been proteins that can be considered 'benign' in that they have no effect on chloroplast metabolism. To-date there has been only a few reported attempts to express membrane-associated proteins, or enzymes that introduce novel metabolic pathways (Wu *et al.*, 2010; Blatti *et al.*, 2012).

As a consequence, *C. reinhardtii* has yet to compete effectively with other, heterotrophic production hosts for the production of recombinant biopharmaceuticals. Here, we have taken the approach that algae may be preferentially suited for the production of plant-specific compounds. One such class of compounds is terpenoids. These are large, structurally-complex plant metabolites that include a range of very high-value examples including paclitaxel, artemisinin and ingenol-3-angelate (Wang *et al.*, 2005). Importantly, their complex structures almost invariably mean that efficient chemical synthesis is extremely difficult, and usually impossible. In principle, it should be possible to introduce novel terpenoid biosynthesis pathways into the plant or algal chloroplast, but this would require re-targeting of the key enzymes in their synthesis, cytochromes P450, into the chloroplasts in an active form. Nielsen et al. have reported the successful synthesis of Dhurrin in tobacco chloroplasts and showed that synthesis is light-driven using native ferredoxin as the electron donor (Nielsen *et al.*, 2013). Dhurrin is a plant defense compound found in *Sorghum bicolor*. While Dhurrin is not a terpenoid, it is synthesised from tyrosine by the means of two ER localised cytochrome

P450 enzymes, CYP79A1 (Sibbesen *et al.*, 1995), CYP71E1 (Kahn *et al.*, 1997) and POR (NADPH cytochrome P450 oxidoreductase), as well as the soluble cytosolic UDP glucosyl transferase UGT85B1 (Jones *et al.*, 1999).

As a first step in introducing such a pathway into a phototrophic microorganism suitable for industrial cultivation (Specht *et al.*, 2010), we report the stable expression of the cytochrome P450 CYP79A1 in the chloroplast of *C. reinhardtii* using a simple transformation method that results in a transgenic line with the CYP79A1 synthetic gene as the only foreign DNA. The enzyme is targeted into the organelle membrane by means of its endogenous N-terminal region, accumulates to 0.4 % of total cellular protein and is shown to be active in the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime. The work paves the way for the introduction of additional components of the Dhurrin pathway.

5.3.3. Results and discussion

5.3.3.1. Transformation strategy and construction of the chloroplast expression vector

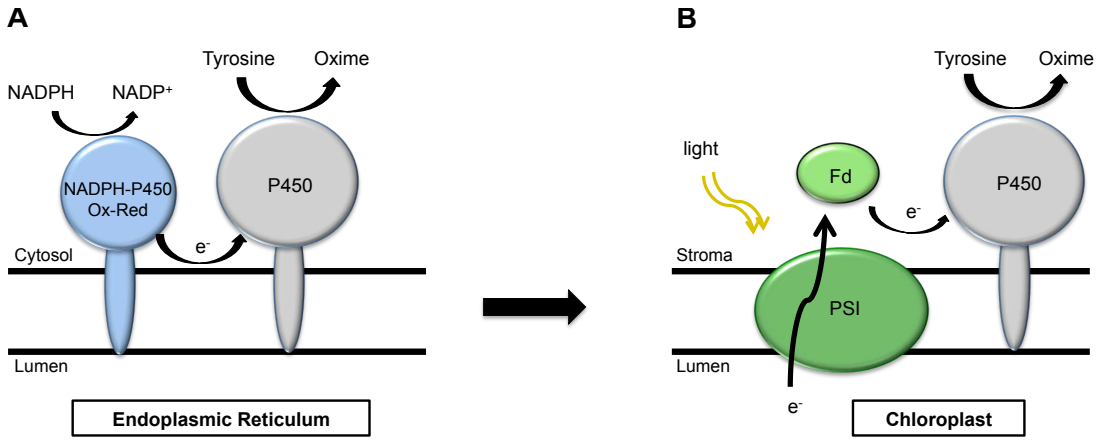

Fig. 5.1. Strategy for driving cytochrome P450 activity by photosynthetic electron transport. **A** Normal action of P450s. Typical P450s have an N-terminal transmembrane span that anchors the protein in the ER membrane. Reducing power is provided by NADPH via NADPH-cytochrome P450 oxido-reductases (NADPH-P450 ox-red). **B** Strategy for driving activity by photosynthetic electron transport: the P450 is synthesised in the chloroplast, targeted into the thylakoid membrane and reducing power is provided by PSI via Fd.

The overall strategy for this study is shown in Fig. 5.1. In the ER, P450s are located in the ER membrane by means of an N-terminal transmembrane span; they drive reactions using reducing power from NADPH but the immediate electron donor is cytochrome P450 reductase (POR). While the chloroplast does contain imported P450s that are involved in carotenoid biosynthesis (Quinlan *et al.*, 2012) the vast majority are located in the ER and we deemed it important to determine whether such proteins can be expressed in chloroplasts and targeted to membranes in an active form. In this study the aim was to express a model P450, CYP79A1, in *C. reinhardtii* chloroplasts, target the enzyme to the thylakoid membrane and drive the reaction using reduced ferredoxin (Fd). This has been shown to be possible in transiently transfected tobacco chloroplasts (Nielsen *et al*., 2013) and our aim was to determine whether algal chloroplasts are a viable production base for stable expression of these enzymes.

A recently developed method for chloroplast transformation (Economou *et al*., 2014) uses a *C. reinhardtii* strain in which the chloroplast *psbH* gene has been replaced by the *aadA* antibiotic resistance cassette. The vector pASapI is used to introduce the gene of interest and restore photosynthetic growth. The expression cassette of pASapI is comprised of the promoter, the 5'UTR and the start codon of *atpA*, a multiple cloning site, and the stop codon and 3' UTR of *rbcL*. Upon homologous recombination the *aadA* cassette is replaced with both a functional copy of *psbH* and the gene of interest (in this case the coding sequence for CYP79A1; see Fig. 5.1). Importantly, the gene of interest remains the only segment of foreign DNA in the transformed genome.

The CYP79A1 coding sequence was synthesised *de novo*, incorporating the coding sequence for a C-terminal HA tag to facilitate identification and analysis. The synthesised CYP79A1 construct was cloned into pASapI as indicated in Fig. 5.2, and *C. reinhardtii* chloroplast transformation was performed by rapid agitation of a DNA/cell mixture with glass beads and selection for photoautotrophic growth. After approximately four weeks transformant colonies were obtained and subjected to further analysis. Control transformations, performed without the addition of DNA, did not yield any colonies.

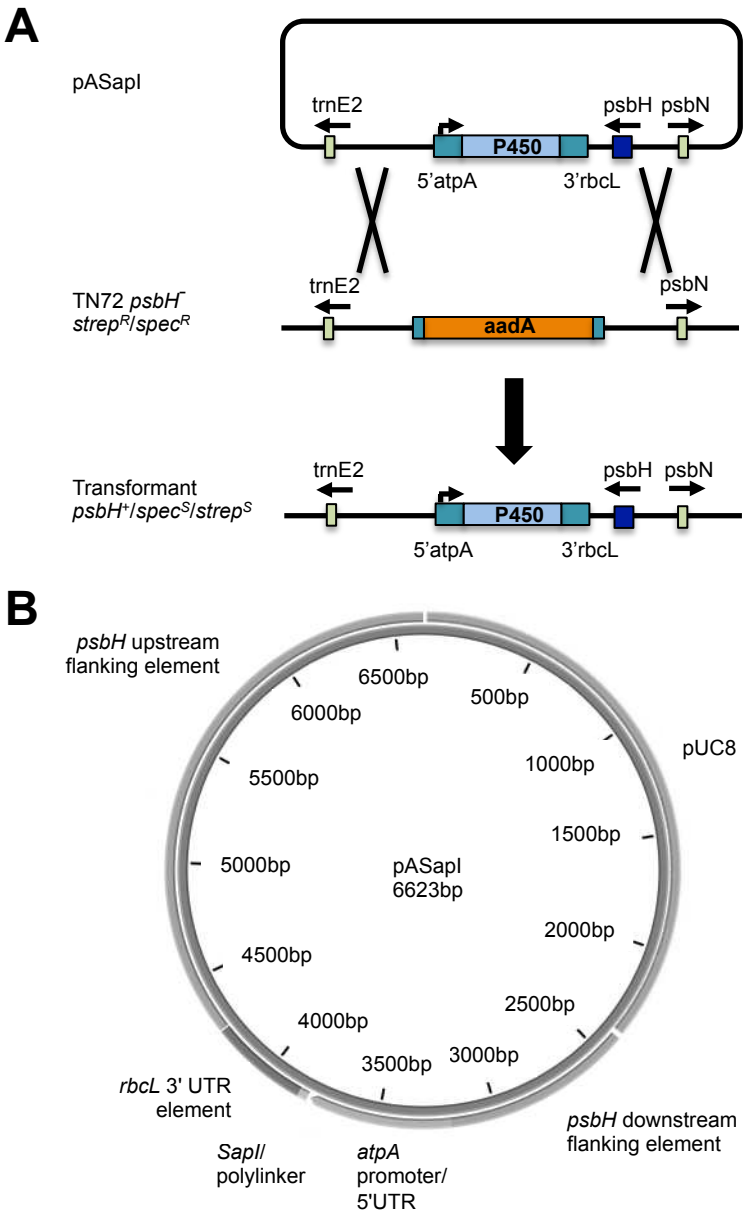

Fig. 5.2. Chloroplast transformation strategy. **A** The vector pASapI uses the essential photosynthesis gene *psbH* as a selection marker for the targeted integration of a gene of interest (in this case *CYP79A1*) into the chloroplast genome. The expression cassette is located in the *psbH-trnE2* intergenic region and contains the promoter, 5'untranslated region and start codon of *atpA*, a multiple cloning site and the stop codon as well as the 3' untranslated region of *rbcL*. An *aadA* cassette replaces *psbH* in the recipient strain rendering it unable to grow on minimal medium lacking acetate. After transformation a functional copy of *psbH* together with the GOI is introduced back into the genome by homologous recombination. **B** pASapI vector design. The pASapI vector was constructed using pUC8 as a backbone. It contains *psbH* flanking elements, the promoter and 5' untranslated region (UTR) of *atpA*, a polylinker with *SapI* and *SphI* sites to introduce the gene of interest and the 3'UTR of *rbcL* (Economou *et al*., 2014).

5.3.3.2. Integration of the CYP79A1 construct into the chloroplast genome

Correct integration of the *CYP79A1* gene into the chloroplast genome was confirmed using a PCR approach. Primers were designed to amplify a 1200 bp fragment from transformed lines (containing an intact restored *psbH* gene).

Fig. 5.3 shows a PCR analysis of the transgenic line (CYP79A1) with the 1200 bp band clearly visible; control PCR reactions using primers for the disrupted *psbH* gene confirmed the homoplasmic integration of the CYP79A1 construct into the chloroplast of *C. reinhardtii.*

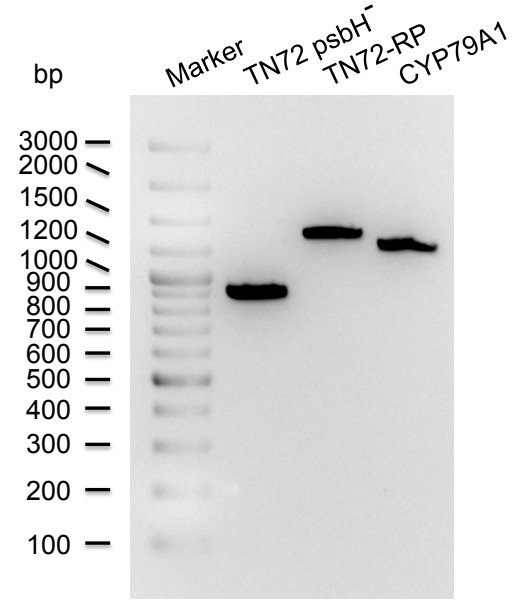

Fig. 5.3. Integration of the CYP79A1 construct. Genomic DNA was isolated from transformed cultures (CYP79A1) and subjected to PCR analysis to test for integration of the CYP79A1 construct. As a control the same was done with untransformed cells (TN72 *psbH*$^-$) and restored wild type cultures (TN72-RP). The figure shows the presence of a 1200 bp band in the transgenic lines, which confirms the integration of the expression cassette into the chloroplast genome. Untransformed cells produce a PCR product of 850 bp length.

5.3.3.3. Accumulation and localisation of the recombinant CYP79A1 protein in the chloroplast

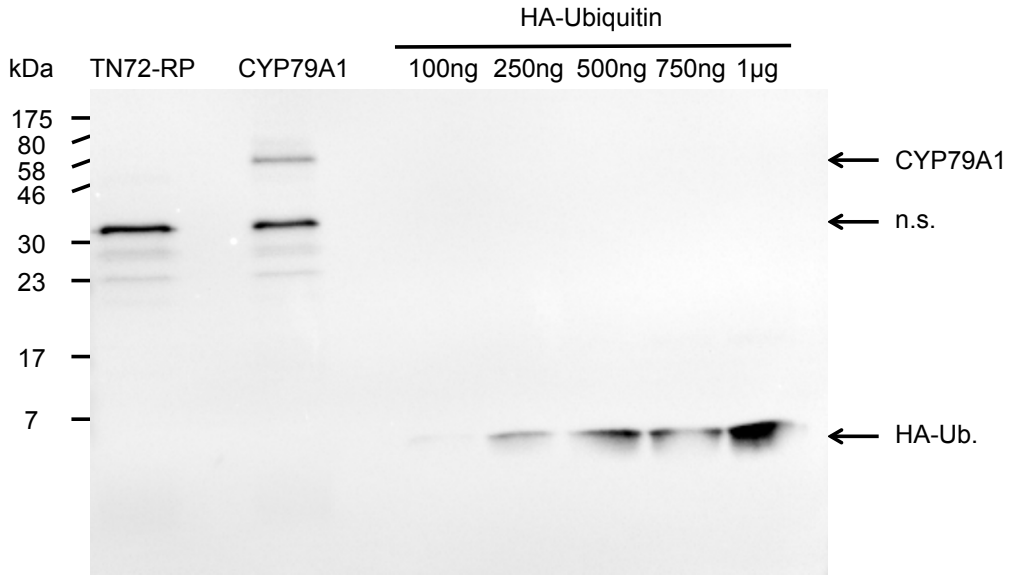

Fig. 5.4. CYP79A1 is expressed in the chloroplast of *C. reinhardtii*. Total cell protein samples of restored wild type (TN72-RP) and transformant strains (CYP79A1) were separated using SDS–PAGE and subjected to immunoblot analysis using an anti-HA antibody. Known amounts of a reference protein (HA-ubiquitin) were loaded on the same gel. CYP79A1 band is indicated; 'n.s.' denotes non-specific cross-reacting band found in both cultures; HA-Ub denotes HA-Ubiquitin.

The expression of *CYP79A1* was tested by immunoblotting of C. *reinhardtii* samples using antibodies to the HA tag as shown in Fig. 5.4. A clear protein signal of 58 kDa, close to the expected size of CYP79A1 (62 kDa) was detected in samples from transgenic cells (CYP79A1 lane) but not in samples from the restored wild type (TN72-RP). The anti-HA antibody also detects a smaller polypeptide, but this is also present in control samples indicating a non-specific reaction (band is denoted n.s.).

To estimate the amount of protein expressed in the chloroplast of *Chlamydomonas* we used a HA-tagged reference protein, HA-Ubiquitin. Fig. 5.4 shows the signals obtained with increasing loadings of this protein and densitometric analysis of calibrated immunoblots revealed an accumulation of approximately 900 ng of protein per mL of culture. We calculated that the expressed CYP79A1 corresponds to 0.4 % of total cell protein.

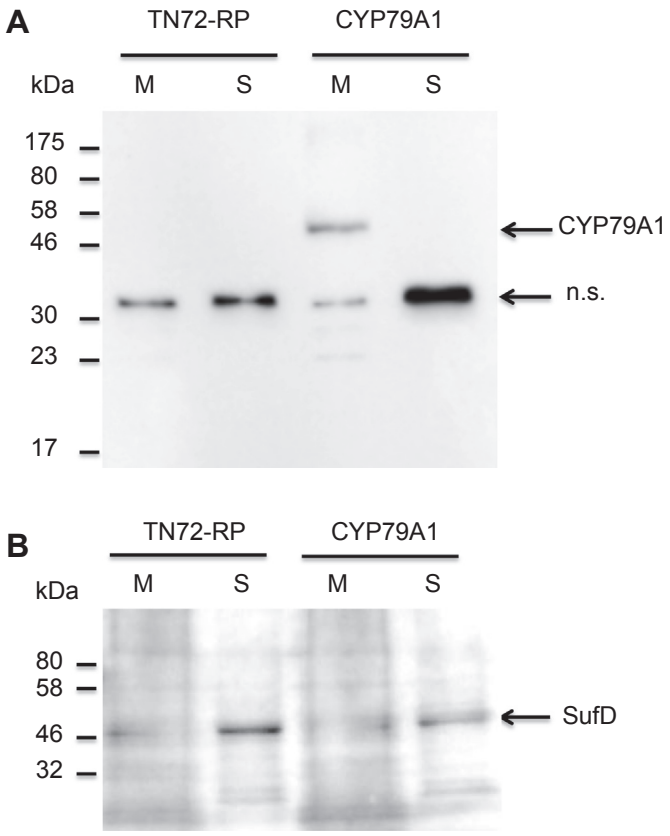

Fig. 5.5. Membrane targeting of CYP79A1. Cells of the CYP79A1 transformant and restored wild type (TN72-RP) cultures were lysed by sonication and the membrane (M) and soluble (S) fractions were separated by ultracentrifugation. Total protein in the fractions was subjected to SDS–PAGE and immunoblotting with an antibody against the HA-tag (**A**) or SufD (**B**). The band corresponding to CYP79A1 is found in the membrane fraction of the transgenic line confirming that CYP79A1 is targeted into the membranes of *C. reinhardtii*. A non-specific cross-reacting band is denoted by n.s.

Since CYP79A1 is a membrane-bound enzyme in its natural environment (the plant ER) we investigated its location in algal cells to confirm membrane targeting and insertion. TN72-RP and CYP79A1 cells were lysed by sonication and the soluble and insoluble fractions were separated by ultracentrifugation. Immunoblotting shows that the CYP79A1 signal is almost exclusively detected in the membrane fraction (lane M in Fig. 5.5 A) of the transgenic cell line. As a control for lysis of soluble proteins, we blotted the same fractions with an antibody to SufD, a component of the chloroplastic FeS cluster synthesis pathway, and Fig. 5.5 B shows this protein to be localised in the soluble fraction as expected. As a control for the pelleting of thylakoids membranes, we confirmed that essentially all of the chlorophyll (over 98 %) was pelleted by this procedure (data not shown). These data show that the CYP79A1 protein is efficiently

targeted into membranes during or after synthesis in the chloroplast by means of its endogenous N-terminal transmembrane span. The data also show that the band generated by non-specific reaction with the antibody (n.s.) is present in the soluble fraction.

5.3.3.4. Enzymatic activity of CYP79A1

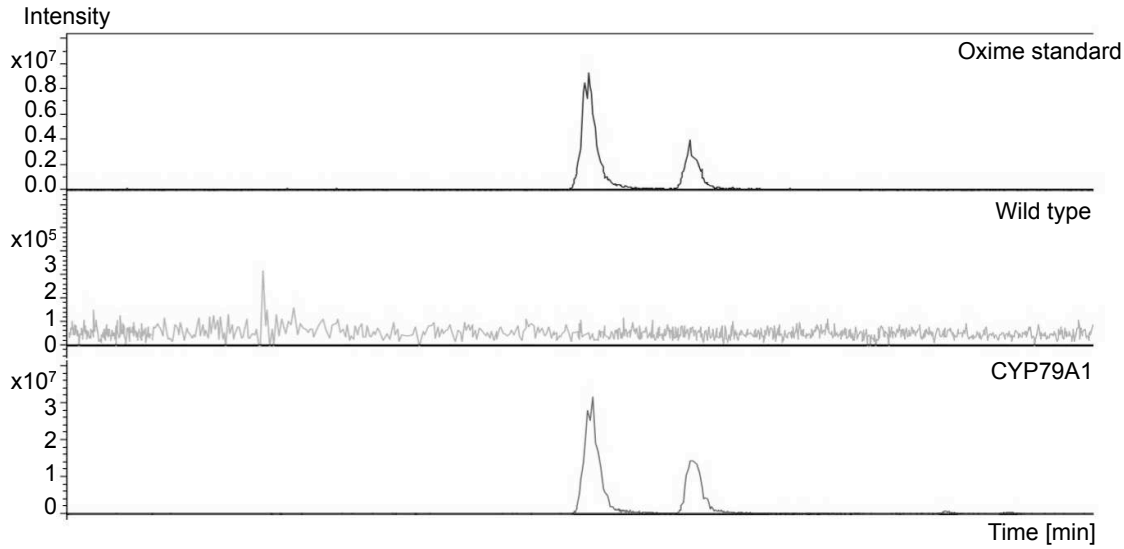

Fig. 5.6. Expressed CYP79A1 is active. The product of CYP79A1, *p*-hydroxy-phenylacetaldoxime, was extracted from the culture medium of CYP79A1 transformant or restored wild type cultures with ethyl acetate after pelleting the cells by centrifugation. The samples were analysed by liquid chromatography–mass spectrometry and compared to an oxime standard as described in Section 5.3.5.7.

CYP79A1 is the first enzyme in the Dhurrin synthesis pathway and it converts tyrosine to *p*-hydroxyphenylacetaldoxime. To test whether the enzyme is active in the *Chlamydomonas* transformant chloroplasts, we subjected samples of the transgenic strain and restored wild type to LC-MS analysis. A comparison of the mass spectrum to that of an oxime standard revealed that the enzyme is active in the CYP79A1 transformant (Fig. 5.6); as expected, the restored wild type sample does not exhibit any oxime-related peaks. Interestingly, the oxime was detected in the culture medium of unlysed transformant cells, but was absent in extracts of both the soluble or insoluble fractions after sonication. This shows that the oxime reaction product is able to exit the

cells, presumably by diffusion since the cells are unlikely to contain an efflux system for this compound. The much smaller volume of the cells (compared to the culture medium) probably explains why the product is not detected in those samples.

5.3.4. Concluding remarks

Cytochrome P450 monooxygenases catalyse key reactions in the biosynthetic pathways of many complex natural products including terpenoids. They are normally located in the endoplasmic reticulum by means of N-terminal transmembrane spans (Frear *et al.*, 1969; Zhao *et al.*, 2014). However, these NADPH requiring pathways usually proceed inefficiently, and plants additionally contain numerous other P450s (typically around 300, although not all are expressed at the same time). A given P450 is usually present at relatively low levels, and, in general, any single P450-dependent pathway usually catalyses low-level synthesis of a given product. It is therefore highly attractive to relocate the cytochrome P450-dependent steps from the ER to the chloroplast of *C. reinhardtii*; the key P450s may then be present in higher amounts and able to use reducing power supplied by photosynthesis to drive their reactions. In this proof of concept study we have tested whether CYP79A1, the first enzyme in the biosynthetic pathway of Dhurrin, can be expressed in the chloroplast membranes of *C. reinhardtii* in an active form. CYP79A1 was chosen because it is easily assayed and has been transiently expressed in tobacco and targeted to the chloroplast (Nielsen *et al.*, 2013). The enzyme has also been expressed in cyanobacteria (Lassen *et al.*, 2014a). The results show that the enzyme is indeed expressed, targeted into membranes and active. The protein levels (0.4 % of total protein) are sufficiently high to readily detect enzymatic activity, the reaction product being detectable in the culture medium where it is relatively dilute.

There are good reasons to believe that the CYP79A1 is driven directly by reducing power supplied by photosynthesis. P450s do not oxidise NADPH themselves, and in the ER, they are driven by a set of specific enzymes termed NADPH cytochrome P450 oxido-reductases, which transfer reducing power from NADPH. When CYP79A1 was expressed transiently in tobacco chloroplasts, it was shown to use electrons from photosynthetically reduced Fd (Nielsen *et al.*, 2013). It is therefore assumed that the

algal-expressed CYP79A1 in this study is similarly driven by reduced Fd, but we should emphasise that this has not been shown directly. The highly negative redox potential of Fd and its high abundance raises the possibility that the cytochrome P450 may work very efficiently in the chloroplast, but we have not compared the specific activities of the ER- and plastid-localised forms and this will be a priority for future studies.

While the primary aim of this study was to test the feasibility of expressing cytochrome P450 enzymes in algal chloroplasts, the results have relevance for the wider field of microalgal biotechnology. To date, chloroplast transformation of *C. reinhardtii* has been used to express soluble proteins such as antibodies or vaccine candidates (Tran *et al.*, 2009; Demurtas *et al.*, 2013), but to our knowledge no membrane-bound protein has been expressed in an active form. Moreover, there are reasons to consider that this may be intrinsically difficult to achieve; the thylakoid and envelope membranes differ radically in lipid composition when compared to other membranes (ca. 80 % galactolipids, whereas the ER is primarily composed of phospholipids). In addition, we assume that the CYP79A1 is targeted into the thylakoid membrane which accounts for the vast majority of chloroplast membrane surface area (although some CYP79A1 may be targeted to the envelope membranes), and proteins are targeted into this membrane by mechanisms that differ in fundamental respects from those used for insertion of proteins into other membranes, including the ER (Woolhead *et al.*, 2001). It was therefore important to confirm that an ER membrane protein can be targeted into chloroplast membranes at reasonably high levels, in an active form.

To summarise, we show here the first relocation of an active, ER membrane-bound cytochrome P450 to the chloroplast of *C. reinhardtii*. A simple chloroplast transformation method and selection strategy was used to create a transformant strain that lacks any antibiotic resistance markers. The enzyme almost certainly uses reducing power from photosynthesis to drive the conversion of endogenous tyrosine to an oxime that appears to diffuse from the cell into the medium. This study thus represents a first step in the proof-of-concept for the production of diterpenoids in the *Chlamydomonas* chloroplast.

5.3.5. Experimental

5.3.5.1. *Chlamydomonas* cultures

Strains were grown in acetate-containing (TAP) medium or minimal (HSM) medium as described in (Harris, 2009), however a revised trace metal recipe was used as found in (Kropat *et al.*, 2011). Cells were grown in 100 mL cultures, at 25 °C, shaking at 110 rpm using illumination at 15 μmol photons m^{-2} s^{-1}.

5.3.5.2. Plasmid design

The pASapI vector, described in Economou et al. (Economou *et al.*, 2014), was used for the introduction of the CYP79A1 coding sequence into the chloroplast genome. The CYP79A1 amino acid sequence (accession number XP_002466099) was codon optimised using the Codon Usage Optimizer (http://codonusageoptimizer.org/down-load/) with codon frequency-, pair- and weight tables of highly expressed chloroplast genes handpicked by the developer. The optimised sequence was synthesised using GenScript (Piscataway, USA). The gene was cloned into pASapI using the restriction enzymes *SapI* and *SphI* (New England Biolabs) to create pASapI- CYP79.

5.3.5.3. Chloroplast transformation

Chloroplast transformation of *C. reinhardtii* was based on a previously described method (Kindle, 1990; Kindle and Sodeinde, 1994) and involved the agitation of an algal/DNA suspension with glass beads of 400–625 μm diameter (Sigma, St Louis, USA). A 400 mL culture grown to early log phase (approx. 2 x 10^6 cells/ mL) was concentrated by centrifugation and resuspended in HSM to 4 mL. 300 μL of these cells (at approx. 2 x 10^8 cells/mL) was added to a sterilised 5 mL test tube containing 300 mg sterile glass beads, followed by 5–10 μg circular plasmid DNA (either pASapI-CYP or the empty pASapI vector as a control). The mixture was agitated vigorously at the maximum speed of a Vortex Genie II (Fisher, Loughborough, UK) for 15 s. The cells were spread on HSM selective agar plates after mixing with 0.5 % molten (42 °C)

agar of the same selective medium. The plates were incubated at 25 °C covered with tissue paper (20 μmol photons m^{-2} s^{-1}) overnight then transferred to a moderate light (50 μmol photons m^{-2} s^{-1}) the next day. Transformant colonies were picked after approximately 4 weeks and restreaked from single colonies three times on selective medium to ensure homoplasmy. The transgenic lines were subjected to PCR analysis to confirm correct integration of the transgene (principle described in (Economou *et al.,* 2014)). A representative line was chosen and termed TN72-CYP79A1, together with a pASapI transformant as a negative control (=TN72-RP).

5.3.5.4. Cell disruption

Culture cell pellets were resuspended in sonication buffer (10 mM Tris-HCl pH 8, 5 mM $MgCl_2$) and lysed by sonication (MSE Soniprep150 plus, amplitude 8 lm, 2 times 20 s with 30 s cooling on ice in-between). The membrane and soluble fractions were separated by ultracentrifugation (70,000 rpm, 1 h, 4 °C, TL- 100 ultracentrifuge, Beckman, Pasadena, USA).

5.3.5.5. SDS-PAGE and immunoblotting

Total chlorophyll content of cultures was determined by extracting chlorophyll with 80 % acetone and measuring absorbance at 645 and 663 nm (Arnon, 1949). An amount of culture equivalent to 5 μg chlorophyll was separated by SDS–PAGE (15 % acrylamide) at 40 mA using a Bio-Rad Mini-PROTEAN® Tetra cell system. Proteins were blotted onto PVDF (BioTrace™, Pall Corporation, Port Washington, USA) membranes using a Bio-Rad Mini-PROTEAN® Tetra cell system according to manufacturer's instructions. The membranes were blocked overnight with 5 % skimmed milk powder (Thermo Scientific, Waltham, USA) in PBS (Formedium, Norfolk, UK) +0.01 % Tween-20 (Sigma, St Louis, USA) (PBS-T) at 4 °C, washed with PBS-T and incubated with anti-HA primary (Sigma, St. Louis, USA) antibody or an antibody to SufD (kindly supplied by Dr. A. Tsaousis, University of Kent) at a concentration of 1:1000 in PBS-T shaking for 1 h at room temperature. After PBS-T washes they were

incubated with an HRP coupled secondary anti-rabbit antibody (1:5000 in PBS-T, Promega) for 1 h shaking at room temperature. The membranes were washed once again and visualised by enhanced chemiluminescence (Clarity Western ECL Substrate, Bio-Rad) according to manufacturer's instructions. The chemiluminescent signal was recorded using a Bio-Rad ChemiDoc XRS+ system. The exposure time was set to 2 min with pictures being taken every 4 s. The marker was recorded and overlaid with a selected blot image.

5.3.5.6. Protein determination

Protein determination was performed using the Bio-Rad DC Protein assay according to manufacturer's instructions. Following cell lysis by sonication and ultracentrifugation, the soluble fraction was taken directly for protein determination. The membrane fraction was first solubilised in 50 mM Tris–acetate pH 8.2, 2.5 mM EDTA pH 8 and 1 % (w/v) SDS and then subjected to the assay.

5.3.5.7. Oxime-extraction and LC-MS analysis

5 mL of transformed and restored wild type cultures were centrifuged to separate cells from the medium. The cell pellet was resuspended in 200 µL 50 mM Tris–HCl pH 8.0 and lysed by sonication (Qsonica, amplitude 100 %, 15 cycles, pulse-on time 30s, pulse-off time 90 s). Broken cells were mixed with 200 µL ethyl acetate and vortexed for 10 min. The samples were then centrifuged at 10000 g for 10 min, the supernatant was collected and evaporated completely. Media samples were mixed with 2 mL ethyl acetate and shaken for 1h. After centrifugation (10000×*g* for 10 min) the upper ethyl acetate phase was collected and evaporated. The dried samples from pellet and media extractions were resuspended in 100 µL 80 % MeOH and 50 µL were mixed with 150 µL Milli-Q water. The samples were then subjected to liquid chromatography–mass spectrometry analysis as described in (Saito *et al.*, 2012). The analysis was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany).

A Zorbax SB-Aq column (Agilent; 3.5 μM, 2.1×150 mm) was used at a flow rate of 0.2 mL min^{-1}. The oven temperature was maintained at 35 °C. The mobile phases were: (A) 2 mM ammonium acetate; (B) methanol. The gradient program was: 0–1 min, isocratic 25 B; 1–11 min, linear gradient 25–60 % B; 11–12 min, isocratic 98 % B; 12–20 min, isocratic 25 % B. The mass spectrometer was run in positive APCI mode and the recorded mass range was *m/z* 80–350.

5.3.6. Acknowledgments

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 317184. This material reflects only the author's views and the Union is not liable for any use that may be made of the information contained therein. The authors also gratefully acknowledge financial support (1) from the VILLUM Center of Excellence ''Plant Plasticity'', (2) from ''Plant Power: Light-Driven Synthesis of Complex Terpenoids Using Cytochrome P450s'' (12-131834) funded by the Danish Council for Strategic Research, Programme Commission on Strategic Growth Technologies. We thank Carl Erik Olsen for the LC-MS measurements.

The authors declare that there is no conflict of interest associated with this work.

Chapter 6: Discussion

In this thesis, several aspects of high-value compound and recombinant protein production in the chloroplast of *C. reinhardtii* have been investigated. In chapter 2, a simple chloroplast transformation method was used to generate stable transformants expressing comparably high levels of a diterpene synthase, TPS4. Homoplasmy of the generated transformants and stable expression levels were demonstrated and the protein identity was confirmed by a two-step purification of the protein and subsequent mass fingerprint analysis. In chapter 5 a further proof of concept is shown investigating the potential of relocating P450s to the thylakoid membranes allowing light-driven product synthesis (see chapter 1.5.2). A well-characterised P450, CYP79A1, was chosen as a model protein and expressed in the chloroplast using the same approach as in chapter 2. It was shown that the enzyme was not only expressed in the homoplasmic transgenic strains, but it was also active *in vivo.* The product, *p*-hydroxyphenylacetaldoxime, was excreted to the culture medium as LC-MS analysis of the culture supernatant showed. Both these studies aimed at setting the scene for development of the *Chlamydomonas* chloroplast as a biotechnological terpene production platform.

In this context, it is crucial to be able to grow the transgenic strains at larger scale. This was investigated in chapter 3. Three *Chlamydomonas reinhardtii* strains, the TPS4 and CYP79A1 transgenic strains and a CW-15 negative control strain were grown in 100L PBRs in a GMO certified greenhouse facility in Lisbon, Portugal. We were able to show that the strains can be cultivated at this scale and stable expression levels of the recombinant proteins were observed, but they exhibited rather poor autotrophic growth capabilities.

In chapter 4 a closer look at novel tools for recombinant protein production in general was taken. Localised expression and targeting of recombinant proteins to the thylakoid lumen were examined using two model substrates – a fluorescent protein, pHRed, and a simple antibody fragment of biopharmaceutical relevance, an scFv. We demonstrated efficient translocation to the thylakoid lumen facilitated by expression with a bacterial Tat export signal peptide.

All studies presented in this thesis aimed at a better understanding of the potential of the algal chloroplast as a host for recombinant protein and high-value compound production. One way of making microalgae a viable biotechnological platform could be to integrate product synthesis within algal metabolism and scavenge intrinsic unique properties such as isoprenoid precursor molecules or coupling of

synthesis to the PET. It will be difficult to compete with existing eukaryotic biotechnological platforms, such as CHO cells or yeast, because the synthesis of conventional recombinant proteins in these organisms has clear advantages. These include higher yields and a longer history of research in process and product optimisation. Thus, for developing a competitive microalgal platform, products of industrial interest will have to take advantage of the unique characteristics of microalgae. Here, potential developments into this direction are shown. How the experiments presented in this thesis are expanding the toolkit of the *C. reinhardtii* chloroplast and how they can be significant for future biotechnological applications will be discussed in more detail in the following sections.

6.1. A proof of concept: terpene production in *Chlamydomonas*

6.1.1. *Cis*-abienol synthesis in *C. reinhardtii*

The synthesis of terpenes is usually a very complex process operated by multi-enzyme pathways. From a genetic engineering perspective, this makes it a difficult task in microalgae because the tools for multiple gene expression are still rare and poorly developed (see Section 1.3.5). The bifunctional diterpene synthase TPS4 from *Abies balsamea* (Zerbe *et al.*, 2012) is therefore a rather unique enzyme as it allows the catalysis of the diterpene, *cis*-abienol, directly from the universal diterpene precursor molecule GGPP. As outlined in Figure 1.4, GGPP should be available in the chloroplast of *C. reinhardtii* and the enzyme had previously been expressed recombinantly in *E. coli* (Zerbe *et al.*, 2012), providing a good setting for a proof of concept study in the *Chlamydomonas* chloroplast.

In chapter 2, a crucial step for *in vivo* diterpene synthesis was described: the stable expression of the recombinant enzyme TPS4 was demonstrated, but activity could not be detected. Purification of the TPS4 enzyme from transgenic algal culture was successful, but the yield was too low for *in vitro* activity assays. Several attempts at extracting the compound directly from the culture and also from lyophilised material with various organic solvents and subsequent GC-analysis remained unsuccessful. Unfortunately, a commercial standard for *cis*-abienol is not available which made product detection very difficult. Later on, the headspace of the transgenic strain was

captured and analysed by GC-MS in collaboration with Kamil Bakowski and Trine Bundgaard Andersen at the University of Copenhagen. An experiment using SPME (Solid-phase microextraction) indicated the presence of *cis*-abienol in the headspace after heating the culture. These data are in line with GC-MS data from a previous *in-vitro* study (Zerbe *et al.*, 2012). Problems with product extraction are not surprising due to the lack of a standardised extraction protocol and most likely very low yields of *cis*-abienol. The low yield can be attributed to several factors including low catalytic activity of the TPS4 enzyme or degradation of the product. Product toxicity seems rather unlikely as no distinct phenotype was observed in the TPS4 strain in comparison with the control (Fig. 1.5, Fig. 3.7B). It is also possible that the GGPP pool is regulated very tightly in the chloroplast limiting the availability of the precursor molecule.

To test the hypothesis of limited precursor availability, a follow-up experiment was developed to increase the GGPP pool in the chloroplast of *C. reinhardtii.* A study by Fukasaki et al. reports the expression of GGPP synthase, from a thermophilic archaeon, in *C. reinhardtii* for potential secondary metabolite synthesis in the alga. However, a thermophilic protein was chosen and at moderate temperatures no additional prenyl transferase activity was found (Fukusaki *et al.*, 2003). A similar approach seems sensible for an increased intra-plastidal GGPP pool, but the enzyme must be able to operate at feasible growth conditions. A genome-based analysis of carotenoid and chorophyll biosynthesis came to the conclusion that GGPP synthesis, via the MEP pathway, is probably limited by the steps catalysed by deoxy-xylulose-5-phosphate synthase (DXS) and geranylgeranyl pyrophosphate synthase (GGPPS) (Lohr *et al.*, 2005). Overexpression of these specific enzymes has previously been shown to improve yields of engineered manoyl oxide, a diterpene, in *Synechocystis* sp. PCC 6803 (Englund *et al.*, 2015). Due to the limited multiple gene expression tools in the *C. reinhardtii* chloroplast, a fusion construct of DXS and GGPPS with a polylinker has been designed and transformants have been shown to express the construct (data not shown), however, no activity was tested yet. This is a first attempt at improving the yield of *cis*-abienol produced in the microalgal chloroplast.

A microalgal production platform for the high-value compound *cis*-abienol is of true industrial interest. *Cis*-abienol can be used for the synthesis of Ambrox®, a synthetic replacement of Ambergris which is widely used in the perfume industry. Currently, Ambrox® is synthesised from sclareol which is extracted from *Salvia sclarea*. However, yields in the plant are very low and can vary with seasonal

conditions. There is evidence that suggests *cis*-abienol is a better and more efficient precursor molecule for chemical Ambrox® synthesis (Barrero *et al.*, 1993) and its potential for metabolic engineering has been highlighted several times (Sallaud *et al.*, 2012; Zerbe *et al.*, 2012). The study presented in chapter 2 offers a solid basis for further improvement of yield and product recovery in order to develop a viable microalgal production platform for *cis*-abienol synthesis.

6.1.2. Paving the way for light-driven terpene synthesis: P450 relocation

The biosynthesis of a terpene typically involves several P450s (Lassen *et al.*, 2014b). Relocation of those enzymes to the chloroplast can facilitate localised and light-driven product synthesis. To date, this has only been demonstrated in tobacco chloroplasts (Nielsen *et al.*, 2013; Gnanasekaran *et al.*, 2015) and cyanobacteria (Lassen *et al.*, 2014a; Wlodarczyk *et al.*, 2016). The study described in chapter 5 shows that this is also possible in eukaryotic microalgae. It has to be highlighted that the model P450 chosen is not of interest for terpene production. CYP79A1 catalyses the rate-limiting step of tyrosine to *p*-hydroxyphenylacetaldoxime as part of the dhurrin pathway, a cyanogenic glucoside found in plants (Busk and Møller, 2002). The extensive accumulated knowledge about this enzyme and the dhurrin pathway combined with the simple transformation method (Economou *et al.*, 2014) enabled straight forward genetic engineering of the chloroplast of *C. reinhardtii*. Not only could the P450 be expressed in the chloroplast and targeted to the membranes (most likely the thylakoids), but an established protocol for oxime extraction and detection also enabled us to demonstrate activity.

The project clearly showed that P450s can be translocated to the chloroplast and thus enable light-driven, localised product synthesis. It paves the way for engineering other P450s of relevance for high-value compound production such as terpenes. Future work will also need to address the issue of P450s co-localisation when several P450s are needed for product synthesis. In the case of CYP79A1, the product was found in the culture medium. If further catalysis of the intermediate is necessary this could constitute a major engineering challenge. It may be worthwhile to explore coupling of P450s that are part of one biosynthetic pathway for a desired product. This might be achievable

via, for example, an engineered shared membrane anchor bringing the P450s in close proximity. To address these advanced engineering tasks, considerable further improvements of the chloroplast toolkit will be necessary. It would also be interesting to further investigate the localisation of the CYP79A1 in the chloroplast. The experiments presented in chapter 5 could not exclude insertion into the envelope membrane although most likely the thylakoid membranes were targeted. Classic labelling experiments could give further insight into this matter.

6.1.3. Implications for *Chlamydomonas* as a terpene production platform

Chapters 2 and 5 have addressed important, basic questions crucial for developing the chloroplast of *C. reinhardtii* for terpene production. The need for a biotechnological terpene production platform is evident; these high-value compounds are of high demand as pharmaceuticals, fragrances, flavours, food supplements and pesticides (Bohlmann and Keeling, 2008). Due to their complexity, chemical synthesis is usually not possible and the compounds have to be extracted from their natural source at low yields and subjected to environmental, seasonal and regional variation (Marienhagen and Bott, 2013). A remarkable success of metabolic engineering was achieved with engineering artemisinin, a sesquiterpene used for malaria treatment in baker's yeast (Ro *et al.*, 2006; Paddon *et al.*, 2013), demonstrating the astonishing potential of microbial platforms for terpene synthesis. A major limitation of terpene synthesis in microbes is that genetic engineering requires a fully elucidated biosynthetic pathway and genetic information. Often terpene pathways have not yet been characterised, thus making a genetic engineering approach impossible. Research into unravelling biosynthetic pathways tackles this problem and will facilitate an increasing diversity of engineered products.

The need for a microbial platform for sustainable and large-scale terpene production is evident given its wide range of potential products. Photosynthetic microbial platforms, such as microalgae, could be an even more attractive host than classical microbial platforms such as *E. coli* or yeast. One reason is their "solar-to-biofuel" generation (Davies *et al.*, 2015) capabilities indicating that very little material input is needed to produce these molecules in a biological system. Additionally, the

cellular context of the production platform chosen has to be considered (Marienhagen and Bott, 2013). Here, microalgae have a clear advantage for recombinant plant terpene synthesis over other classical microbial production systems as they have similar metabolic pathways to higher plants that play a role for terpene synthesis. However, the limited availability of engineering tools in photosynthetic hosts is still a major drawback. With advanced genetic tools available, *C. reinhardtii* is therefore one of the most sensible engineering targets at the moment.

In this thesis several aspects paving the way for terpene synthesis in *C. reinhardtii* were addressed. These confirmed the potential of microalgae for terpene production. Future research will be needed to address low yields, metabolic flux regulation and tackle further biosynthetic pathways of interest for biotechnological applications to unravel the full potential of this photosynthetic microbial host.

6.2. Sophisticated recombinant protein production: expanding the spectrum by protein targeting.

No matter what high-value product the engineering target is, a secondary metabolite or a protein itself, a sophisticated system for protein expression, stability and targeting is of great importance. Tools for protein expression have advanced remarkably (see Section 1.3.). Therefore, we decided to address more basic questions of protein targeting in *C. reinhardtii* with the aim of expanding the chloroplast toolkit. In chapter 4 a closer look at the thylakoid lumen was taken which no study has yet, to my knowledge, utilised for biotechnological applications. The thylakoid lumen is an exciting compartment that surprisingly little is known about. Its significance for photosynthesis regulation is undoubted (Järvi *et al.*, 2013), but further physiological functions have only been hinted at, for example with the help of proteomics (Kieselbach and Schröder, 2003). The thylakoid lumen could provide a novel environment for recombinant protein expression that is rather different from classical expression in the stroma.

The study described in chapter 4 shows that translocation to the thylakoid lumen in the chloroplast of *C. reinhardtii* can be facilitated using a bacterial Tat export signal peptide, ‘TorA’, as a N-terminal leader peptide. Two model substrates, an scFv and a

fluorescent protein were translocated. The translocation of TorA-scFv is promising for looking at further substrates of biotechnological relevance that might, for example, need a lower pH, as typically found in the lumen. The lumen environment can also provide a higher level of protection and increase stability for substrates of a very delicate nature. Admittedly, the imported pHRed is not a biotechnologically relevant protein as the scFv is. However, a proof of concept requires an iterative approach and fluorescent proteins are useful tools to test a novel system as this one is. pHRed is not an ordinary fluorescent protein, it was made by mutagenesis from mKeima enabling *in vivo* pH detection (Tantama *et al.*, 2011). This is a very interesting tool for *in vivo* pH measurements in *C. reinhardtii*. Unfortunately low expression levels and chlorophyll autofluorescence in the transgenic strains hindered further investigations with conventional confocal imaging tools that were available to us. A customised setup with more precise (single wavelength) excitation lasers and a more sensitive detection system could, however, allow *in vivo* measurements in the generated strains. These measurements would be of interest for fundamental research far beyond the scope of this proof of concept study.

The study shown in chapter 4 specifically targets translocation to the thylakoid lumen via the Tat pathway, but this is only a first attempt at exploring the potential of utilising protein targeting for biotechnological applications in microalgae. Depending on the nature of the substrate and the compartment targeted, other translocation pathways will be of interest. In this case, the limiting factors are not necessarily genetic engineering tools but the lack of knowledge regarding the translocation machinery in microalgae. For example, it is not even entirely clear what drives translocation via Tat to the thylakoid lumen. Initially, the process was though to be ΔpH dependent (based on mainly in *vitro* studies), but an *in vivo* study in *C. reinhardtii* suggested no ΔpH dependence in the thylakoids (Finazzi *et al.*, 2003) and later on in tobacco protoplasts the process was found to be also ΔΨ independent (Di Cola *et al.*, 2005). This is only one example showing that progress in genetic engineering and a better understanding of microalgal cell biology and physiology will be of greater importance with increasing complexity of the engineered systems. Otherwise, our knowledge of the intrinsic properties of the engineering target can become the limiting factor for advancing microalgae for biotechnological applications.

6.3. Scalability is crucial for industrial interest

Recombinant proteins and other products expressed and synthesised in *Chlamydomonas*, outlined in Section 1.4.1 and 1.4.2, are very diverse and at a first glance seem impressive and promising. Most of these studies aim at or at least mention the significance of the finding for biotechnological applications or so-called "molecular farming". However, there is a clear gap between the potential shown in proof of concept studies and the transition of these to scale. If the strains cannot be cultivated or produce the product in an industrial setting, it is not a viable system and the implication for its use in biotechnology cannot be justified. In the case of *Chlamydomonas* this missing link between lab scale studies and scale up is particularly noticeable. Only one study has reported the growth of *Chlamydomonas* at pilot scale (Gimpel *et al.*, 2015b) which can be considered a first test of scalability. One reason for this gap might be that *Chlamydomonas* is not an "ideal" organism for an industrial setting – its flagella and low temperature tolerance (Hlavova *et al.*, 2015) make it a less robust organism compared to other microalgae that are already successfully cultivated at industrial scale. Given its potential for genetic engineering and the array of modified strains available it is however crucial to examine ways of making *Chlamydomonas* scalable and to also know the limitations of its scalability. Therefore we tested our genetically modified strains, expressing CYP79A1 and TPS4, at pilot scale in 100L PBRs as shown in Chapter 3. The marker-free engineering approach of these strains (see Section 1.3.5) makes it particularly attractive for scale-up as no antibiotics are necessary to sustain gene expression. This is not only beneficial for cutting costs but also important to minimise environmental hazards. A disadvantage is, however, the wall-deficient nature of the strains resulting in a higher degree of fragility. Given the fact that most engineered *C. reinhardtii* strains are based on wall-deficient mutant strains it was reassuring that, without a thorough upscaling optimisation, we successfully cultivated the strains in the 100 L PBRs. This also implies that considerable improvement might be possible and, at least at a semi-industrial pilot scale, the strains can be cultivated. The next step would have to involve a larger culture volume although the logistics of this operation present several problems. Bigger cultivation vessels would have to be enclosed due to the GM nature of strains. The PBRs used in our study cannot be used at much bigger volumes due to limitations of various kinds (for example light penetration

and aeration). To increase the scale in closed PBRs, a tubular reactor design could be beneficial. These however require the operation of pumps that would potentially cause too much shear stress to the fragile algae. This can be overcome by utilisation of low-shear pumps or innovative flat panel designs (Tredici *et al.*, 2015). Nonetheless, growth of these strains at 100 L scale is promising and might even be sufficient for several applications of these microalgae.

The production of high-value compounds, such as recombinant proteins or terpenes, does not necessarily require the production of a large amount of biomass and cultivation costs are a much less important factor than for low-value commodity production. A system with a scale-out rather than scale-up process might be, especially for systems like diterpene production, thoroughly viable. 'Scale-out' could be achieved by a modular unit design where a larger culture volume is handled by increasing the number of modules instead of increasing the volume of the culture unit ('scale-up'). Some targets to be produced in these microalgal systems have market values over 10 Million EUR per kg (Verdelho Vieira, 2014) making it a very attractive target of further research and transition to industry.

6.4. The toolkit of the *Chlamydomonas* chloroplast: status and perspectives

The *Chlamydomonas* chloroplast has attracted a lot of interest for biotechnological applications in the last years. In this thesis, several proof of concept studies are presented that aim at expanding the already existing capabilities of this toolkit and also at making it industrially relevant. Several aspects of developing a sustainable photosynthetic platform for diterpene production have been investigated and a first pilot scale project also validated the transition into a more industrial setting. All experiments presented aimed at using simple and easily reproducible techniques that are available to a broader research community. A straightforward protocol was used for transformation that does not need any expensive equipment other than common facilities found in a standard molecular biology lab. Marker-free, genetically stable strains were generated that do not need to be supplemented with antibiotics. The proof of concepts confirmed that the development of the *C. reinhardtii* chloroplast for

diterpene production is reasonable and justified, the prerequisites set in this thesis. A simple approach of utilising a bacterial export signal peptide for lumen targeting also showed that the chloroplast still has a lot of untapped potential. Further work, exploring novel ways of utilising the chloroplast, is to be expected.

Although all the experiments presented here are at the proof of concept stage there is good reason to believe that this is a further important step in the right direction of developing microalgae for biotechnological applications. It will now be crucial to build on the knowledge acquired and to integrate the different studies. This could pave the way for improving yields of products already produced in microalgae, enable the engineering of further interesting high-value compounds and ensure the scalability of the system which will facilitate the transition into an industrial setting. A stepwise approach to tackle limitations of the current microalgal systems will help to improve the platform gradually and hopefully, also make it a viable system that can compete at the same pitch with established production platforms.

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